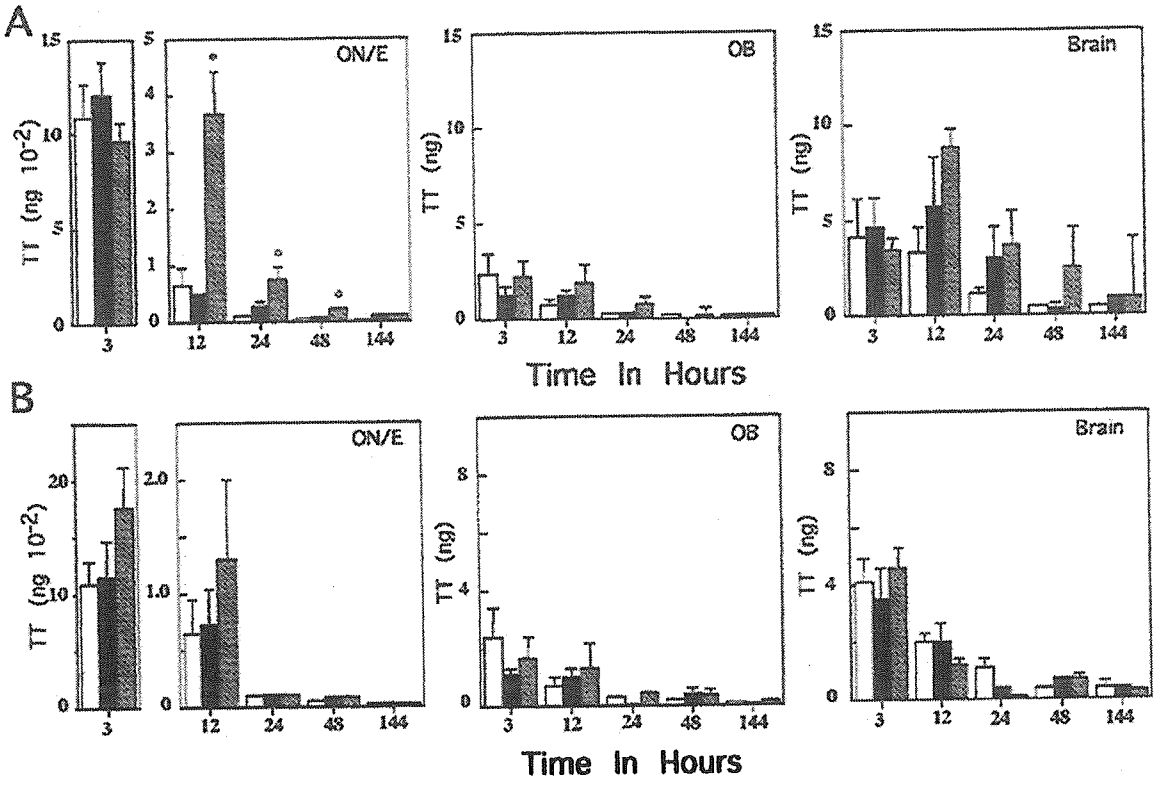

FIG. 2. Comparison of nCT- and mCT- (A) with nLTh-1- and mLTh-1-induced uptake (B) of ^{125}I-TT into olfactory and neuronal tissues. (A) Distribution of ^{125}I-TT in the ON/E, OB, and brain after nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mCT (solid bars) or 1.0 μg nCT (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. (B) Nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mLTh-1 (solid bars) or 1.0 μg of nLTh-1 (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. A total of 20 μg of ^{125}I-TT in 12 μl was given nasally either with or without enterotoxin (6 μl/nare). Significant differences between the ^{125}I-TT-only group and ^{125}I-TT-plus-enterotoxin group are indicated by an asterisk and mark *P* values smaller than 0.05. The averages of 4 to 10 mice plus 1 standard error of the mean are depicted.

exudate from the nares was collected in microcentrifuge tubes. Cells and debris were removed by a 10-min 10,000 × *g* centrifugation step. All samples were frozen at −80°C until they were analyzed by enzyme-linked immunosorbent assay (ELISA). Lymphoid and neuronal tissues were isolated as described previously (46).

Cytokine ELISA. The detection of the cytokines IL-6, IL-1β, and tumor necrosis factor alpha (TNF-α) in plasma and nasal washes was performed on Maxisorp 96-well immunoplates (Nunc A/S, Roskilde, Denmark). The plates were coated overnight at 4°C with the following capture monoclonal antibodies: rat anti-mouse IL-6 (clone MP5-20F3; BD PharMingen, San Diego, CA), rat anti-mouse IL-1β (clone 30311.1; R&D systems, Minneapolis, MN), and hamster anti-mouse TNF-α (clone TN3-19.12; BD PharMingen) at 2 μg/ml. The plates were washed with PBS-Tween 20 (0.05%) and blocked with 1% bovine serum albumin in PBS-Tween 20 (0.05%) for 1 hour at room temperature. Subsequently, the samples were added to 96-well plates and incubated overnight at 4°C. The plates were washed with PBS-Tween 20, and the biotinylated capture monoclonal antibodies rat anti-mouse IL-6 (clone MP5-32C11; BD PharMingen), goat anti-mouse IL-1β (R&D Systems), and rabbit anti-mouse TNF-α (BD PharMingen) at concentrations of 0.5 μg/ml, 300 ng/ml, and 0.5 μg/ml, respectively. For detection of IL-1β and TNF-α, streptavidin-conjugated to horseradish peroxidase (Life Technologies Inc., Rockville, MD) was used at a 1:2,000 dilution and anti-biotin-horseradish peroxidase at a 1:2,000 dilution (Vector Laboratories, Burlingame, CA) was used for IL-6. The ELISA plates were washed, followed by a 15-min incubation with 2,2′-azino-bis-(3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma Chemical Co., St. Louis, MO). The absorption at 415 nm was measured at various sample dilutions, and the cytokine levels were determined using standard curves. The detection limits of the ELISA for IL-6, IL-1β, and TNF-α were 10, 1, and 12 pg/ml, respectively.

Statistics. The data are expressed as the mean plus 1 standard error of the mean, and the results were compared by the two-tailed, unpaired Mann-Whitney or Student *t* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for MacIntosh computers.

RESULTS

Redirection of TT into the ON/E. In order to define the parameters involved in redirection of vaccine proteins into olfactory tissues by nCT and nLTh-1, the role of ADP-ribosyltransferase activity in antigen redistribution was first analyzed by comparison with nonenterotoxigenic mutants of CT (E112K) and LTh-1 (E112K). The presence of ^{125}I-TT in the ON/E, OB, and brain was assessed after nasal application of ^{125}I-TT only or in combination with nCT or mCT (E112K) (Fig. 2A) or with nLTh-1 or mLTh-1 (E112K) (Fig. 2B). The enterotoxin dose was based on the amount required to induce strong mucosal immune responses after nasal application. No significant differences were observed between the distribution of TT protein when given alone or with nCT (1 μg), mCT (10 μg), nLTh-1 (1 μg), or mLTh-1 (10 μg) in all tissues tested, with the exception of the ON/E. Strikingly elevated levels of TT protein were present at 12, 24, and 48 h in the ON/E (P = 0.004, P = 0.028, and P = 0.043, respectively) when given

nasally with nCT. However, minimal uptake was seen at these time points when TT was given alone or with mCT, nLTh-1 (1 μg), or mLTh-1 (Fig. 2A and B). Although the elevated redistribution of ^{125}I-TT into the ON/E induced by nCT was reproducible, it is unlikely that the 48-h time point was of biological importance, since the differences between the groups were relatively small. The nCT-versus-mCT results clearly show that ADP-ribosyltransferase activity is required for redirection of TT into the ON/E. However, it was noteworthy that nLTh-1 (1.0 μg) did not induce significant increases of TT protein in the ON/E at 12 h following nasal application. This TT accumulation was considerably lower than that seen with 1.0 μg of nCT and indicated that factors in addition to ADP-ribosyltransferase activity also play roles in protein redistribution. It should be noted that no preferential accumulation of TT was observed in the OB versus the brain as previously reported for CT-B and CT (46). Thus, unlike CT or CT-B, limited or no axonal transport of TT along olfactory neurons takes place after nasal application, and the distribution of TT in the OB and brain can be explained by the sizes of the organs and the blood associated with them.

Since nCT and nLTh-1 display different ganglioside binding specificities, where nLTh-1 binds in addition to GM1, as reported for nCT and also GD1b, GM2, asialo-GM1, and other intestinal glycoproteins (12, 18, 25), it remained possible that GM1 binding by the enterotoxin was required for protein redirection into the ON/E. Thus, decreased binding by nLTh-1 to GM1 may be due to competition by other nLTh-1 receptors in the nasal tract, which would inhibit protein redirection. In addition, it should be noted that the uptake of TT into various tissues, when 1 μg nLTh-1 was used, was about half of the total uptake observed when 1 μg of nCT was given nasally. To test the GM1 dependence of TT redirection, a dose-response experiment with nLTh-1 was performed (Fig. 3A). Increasing levels of nLTh-1 induced enhanced TT redirection, and this required between a two- and fivefold-higher dose to induce levels not significantly different from those seen with 1 μg of nCT. This observation is consistent with a requirement to target GM1 in order to redirect protein into the ON/E. A comparison between the abilities of nCT, mCT, nLTh-1, and mLTh-1 to redirect protein into ON/E was made (Fig. 3B). In conclusion, nCT was more potent than nLTh-1 in redirecting codelivered protein into the ON/E, while the nontoxic mCT or mLTh-1 was only marginally able to do so at the levels tested.

Distribution of TT in lymphoid tissues. After nasal application of ^{125}I-TT, the NALT, blood, spleen, and CLNs were isolated and analyzed for the distribution of protein when given alone or in combination with nCT, nLTh-1, mCT (E112K), and mLTh-1 (E112K). No significant differences were seen in these lymphoid tissues with the exception of NALT. A significantly decreased accumulation of TT was seen in NALT of mice given TT nasally with nCT (Fig. 4). A small decrease in TT protein accumulation in NALT was observed with mCT, and no significant differences were seen between nLTh-1 and mLTh-1 compared with TT given alone. The data from these observations are summarized in Table 1 and were compared with the observed immune responses induced by these enterotoxin-based mucosal adjuvants. It was interesting that only decreased antigen accumulation in NALT was observed when a strong Th2 response was induced.

The role of gangliosides in protein redirection. In order to assess the role of GM1 ganglioside binding by nLTh-1 and nCT for ^{125}I-TT redirection into the ON/E, the ganglioside binding site was blocked by prior incubation with a 15-fold molar excess of GM1. Blocking the ganglioside binding site of nLTh-1 and nCT with GM1 significantly inhibited redirection of ^{125}I-TT into the ON/E for both 5 μg of nLTh-1 ($P = 0.04$) and 1.0 μg of nCT ($P = 0.02$) 12 h after application (Fig. 5). Blocking the ganglioside binding site also elevated TT levels in the blood, spleen, and CLNs. A significant increase in TT accumulation was observed in the CLNs (which drain the nasal tract) 12 h after application with nLTh-1 (data not shown). Whether this increase of protein in the CLNs was due to a lack of ON/E targeting and resulted in subsequent drainage into the CLNs or was due to enhanced circulation in the blood, or a combination of the two, cannot be distinguished.

In order to determine whether binding to gangliosides other than GM1 would prevent antigen redirection into the ON/E, the heat-labile enterotoxin from serogroup two, LT-IIb, was used. This enterotoxin displays high-affinity binding to GD1a and GT1b and weak affinity for GM3 and does not bind at all to GM1 (12). Furthermore, LT-IIb is more toxic to Y1 adrenal cells than nCT based upon morphological changes and adenylate cyclase activation (24). When the ability of the LT-IIb enterotoxin was compared with that of nLTh-I for redirection of TT into the ON/E, it was very apparent that 5 μg of LT-IIb, unlike nLTh-I, was unable to redirect ^{125}I-TT into the ON/E and did not affect the TT distribution pattern observed in other tissues (Fig. 6). Thus, GM1 targeting appears to be an essential step in directing the ADP-ribosyltranferase to cause codelivered antigen redirection. As pointed out in Fig. 2, the TT associated with the OB was considerably lower than that observed in the brain and reflected the smaller size and lower amount of blood associated with these organs and argues against considerable axonal transport of TT from the nasal tract into the OB as observed with labeled CT and CT-B (46).

Differential production of inflammatory cytokines in the nasal tract. To determine if nasal application of mucosal adjuvants induces inflammatory cytokines, nasal washes and plasma were collected at various time points after nasal delivery. The nasal washes and plasma were analyzed for IL-1β, IL-6, and TNF-α. Differential expression of IL-6 was seen in the nasal washes (Fig. 7). Both nCT- and mCT-treated mice displayed IL-6 levels significantly elevated over those seen when TT was given alone (Fig. 7). Although the levels of IL-6 at 6 h were twofold higher in the nCT- than in the mCT-treated mice, no significant differences were seen between these groups until 12 h after application ($P = 0.026$). Markedly lower levels of IL-6 and IL-1β were seen in the plasma of the mice. The time frame between 3 and 12 h following administration of nCT and TT, when redirection of TT into the ON/E was observed (Fig. 2A), also represented the time when maximal IL-6 secretion was noted in nasal washes. Thus, local inflammatory responses were induced by nCT, and to a much lesser extent by mCT, during this time period. No detectable levels of TNF-α were observed in either plasma or nasal washes (data not shown), and IL-1β levels did not differ significantly among the three groups. These differences in production of IL-6 were not due to the differences in lipopolysaccharide, since the nCT contained ≤0.048 ng/μg and the mCT contained ≤1.0 ng/10

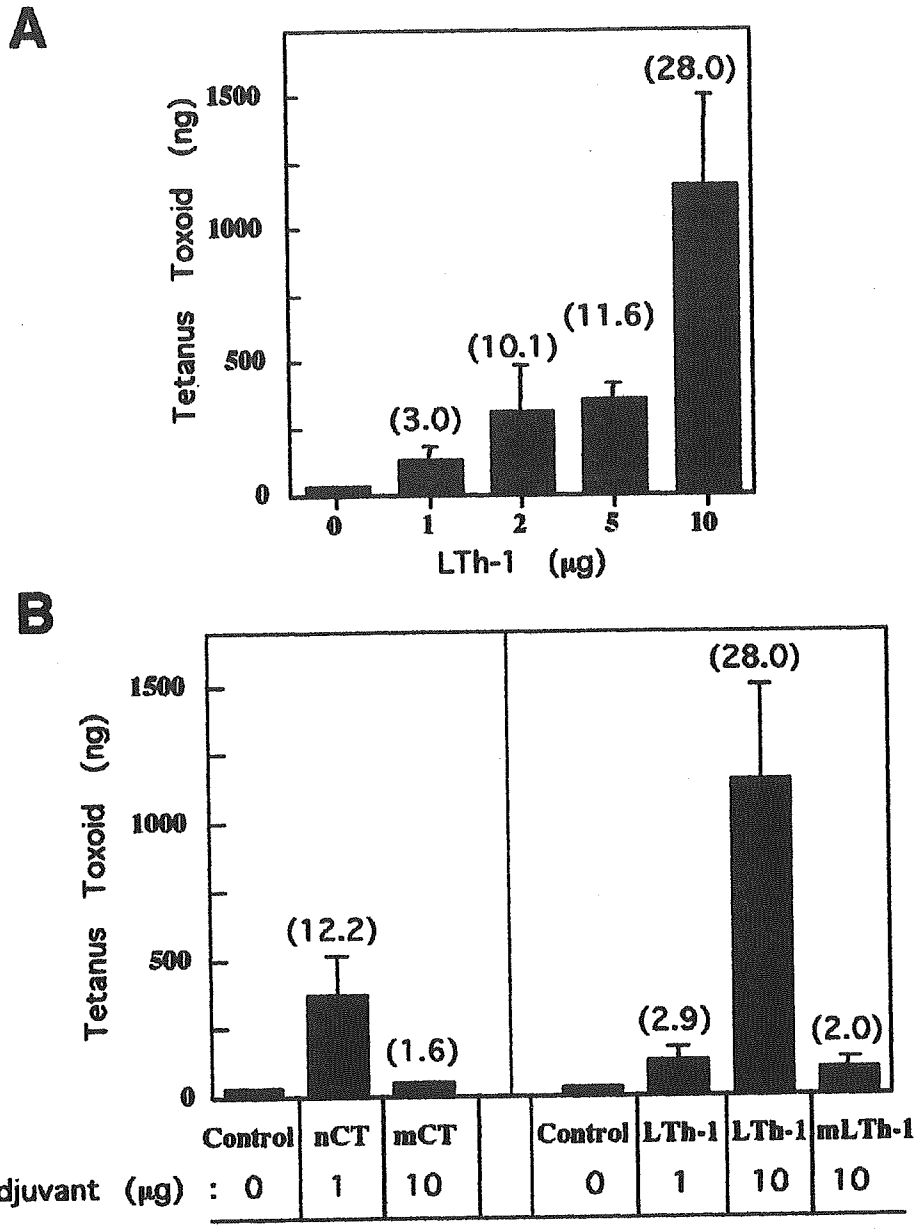

FIG. 3. Enterotoxin-dependent redirection of ^{125}I-TT into the ON/E. Various doses of nLTh-1, i.e., 0, 1, 2, 5, and 10 μg, were combined with 20 μg ^{125}I-TT and applied nasally. The ON/E were collected 12 h after application to assess redirection of the protein. (A) Indicated is the increase (*n*-fold) over control values, i.e., ^{125}I-TT alone, when administered with nLTh-I. (B) Comparison of the degrees of redirection of ^{125}I-TT into ON/E observed with nCT, mCT (E112K), nLTh-1, and mLTh-1 (E112K). Indicated are the means plus standard errors of the mean.

μg. The observations for IL-6 were confirmed by real-time reverse transcription-PCR on RNA derived from the ON/E (data not shown).

To determine whether LTh-1 had similar effects on IL-1β and IL-6 production, nasal washes and plasma were collected from mice treated 12 h prior with TT alone or TT with 1.0 or 5.0 μg of LTh-1. A significant increase in IL-6 was seen in nasal washes when 5 μg of LTh-1 was given with TT, while no significant increase was observed in plasma (Fig. 8). Elevated secretion of IL-6 was also seen in nasal washes with the 1.0-μg LTh-1 dose; however, this increase in IL-6 was not significant compared with TT alone.

DISCUSSION

The redirection of TT protein by native enterotoxin-based mucosal adjuvants raises questions regarding both safety and the molecular mechanisms involved. In this study, we addressed the parameters influencing redirection of the vaccine protein TT into the olfactory nerve/epithelium, NALT, and related lymphoid tissues, as well as the associated production of inflammatory cytokines in the nasal tract. To redirect nasally coadministered ^{125}I-TT into the ON/E by enterotoxin-based mucosal adjuvants, ADP-ribosyltransferase activity is clearly required. This is based upon the finding that both nCT and

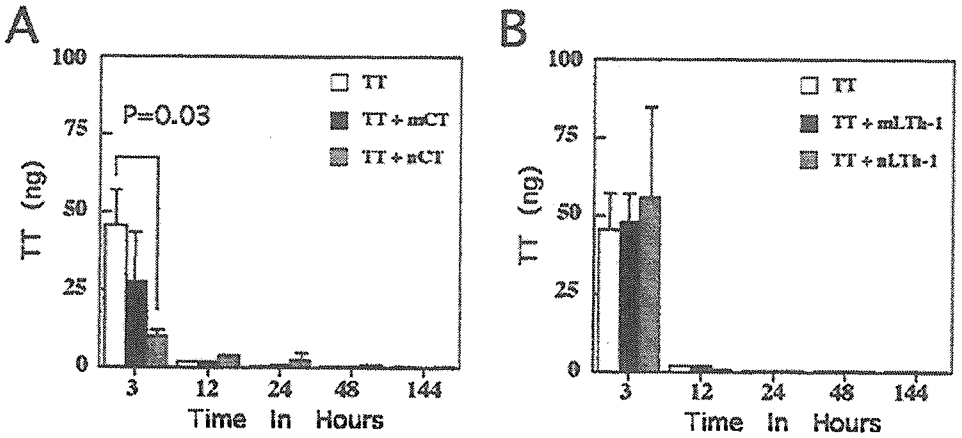

FIG. 4. Comparison of nCT- and nLTh-1- with mCT (E112K)- and mLTh-1 (E112K)-induced uptake of ^{125}I-TT into NALT. Distribution of ^{125}I-TT in NALT is expressed as TT (ng) accumulation per organ. A total of 20 μg of ^{125}I-TT alone or in combination with 10 μg of mCT or 1.0 μg of nCT (A) or ^{125}I-TT alone or in combination with 10 μg of mLTh-1 or 1.0 μg nLTh-1 (B) was given nasally (6 μl/nare). Accumulation of ^{125}I-TT was analyzed at various time points after application. The average of 5 to 10 mice plus standard error of the mean per data point are depicted.

nLTh-1 redirected protein into the ON/E while mCT (E112K) and mLTh-1 (E112K), which both lack ADP-ribosyltransferase activity (28, 54), did not. Thus, ADP-ribosyltransferase activity of the A subunit is an essential, although not sufficient, element for redirection of protein into the ON/E by AB_5 enterotoxins. Binding to GM1 by the B subunit, in addition to ADP-ribosyltransferase activity of the A subunit, also appears to be a prerequisite for redirection of protein into the ON/E, since incubation of nCT or nLTh-1 with excess GM1 prior to administration prevented accumulation of ^{125}I-TT in the ON/E.

The finding that LT-IIb does not redirect TT into the ON/E while it retains full ADP-ribosyltransferase activity and functions as a mucosal adjuvant when delivered nasally (34) may be explained by a requirement for GM1 binding by the enterotoxin to induce protein redirection, since LT-IIb, unlike nCT or nLTh-1, does not bind to GM1 gangliosides (12, 38). Using a human intestinal epithelial cell line (T84), others found that both nCT and LT-IIb bound with high affinity (2 to 5 μM) to the apical membranes of T84 cells (48). However, only nCT was able to elicit a cyclic-AMP-dependent secretory response. Moreover, while nCT-GM1 fractionated with a caveola-like, detergent-insoluble membrane fraction, the LT-IIb-GD1a complex was solubilized by 1% Triton X-100. The authors suggested that signal transduction may require the formation of caveola-like structures and demonstrated that the chimera composed of the LT-IIb A subunit and CT-B was capable of inducing a secretory response. Native CT binding to polarized epithelial cells takes place on the apical membrane surface but targets a basolaterally located effector molecule, i.e., adenylate cyclase (31). It could be hypothesized that the requirement to bind GM1 is associated with the endocytotic pathway taken following GM1 endocytosis. The LT-IIb binds to GD1a, a ganglioside that is not located in the caveola-like membrane domains as reported for GM1 (48), and may thus follow a different intracellular path in epithelial cells. As a consequence of this, the A1 subunits or the ADP-ribose-Gsα may not reach the adenylate cyclase located in the basolateral domain of polarized epithelial cells. This could be an important step for enhanced permeability of the epithelium and the ability of antigen to cross the nasal epithelial barrier.

The nasal tract is covered by a pseudostratified epithelium. Underneath this epithelium, a dense network of fenestrated capillaries provides a readily available blood supply (14). The nasal administration of enterotoxin-based adjuvants would target this epithelium through GM1 ganglioside binding. Our observations and those of others reporting the use of rabbit mucosa in vitro (14) clearly raise questions regarding the safety of nasal use of these adjuvants in humans. Human studies performed previously using nasal nLTh-1 and CT-B suggest that precautions need to be taken when applying these mole-

TABLE 1. Antigen-specific immune response and antigen distribution in NALT following nasal immunization

Adjuvant	Antigen	Plasma IgG[a]	S-IgA[b]	T helper activity	TT in NALT[c]	Reference(s)
	TT	+[d]	+	Th1/Th2	++++	30, 47, 51
nCT	TT	++++	+++	Th2	+	47, 50, 51
mCT(E112K)	TT	++++	+++	Th2	++	50, 51
nLTh-1	TT	++++	+++	Th1/Th2	++++	2, 7, 42
mLTh-1(E112K)	TT	++++	+++	Th1/Th2	++++	2, 7
LTIIb	AgI/II[e]	++++	+++	Th1/Th2	++++	34

[a] IgG, immunoglobulin G.
[b] S-IgA, mucosally-derived secretory IgA.
[c] Data were obtained in this study and indicate the relative TT antigen accumulation in NALT.
[d] +, low-, ++, medium-; +++, high-; ++++, very high.
[e] AgI/II, Antigen I/II from *Streptococcus mutants*.

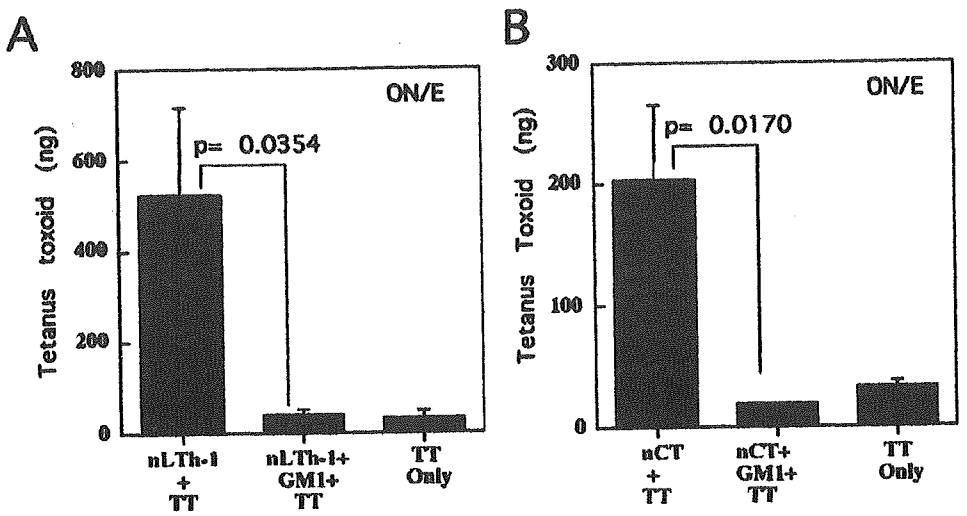

FIG. 5. Influence of blocking the GM1 binding site on nLTh-1 and nCT on tissue distribution of coadministered ^{125}I-TT in the ON/E after nasal application. nLTh-I (A) and nCT (B) were preincubated with a 15-fold molar excess of GM1 for 30 min at room temperature prior to nasal application together with ^{125}I-TT. The cpm associated with the ON/E 12 h after application were analyzed and compared with application without preincubation with GM1 and with administration of ^{125}I-TT antigen alone. A total of 20 μg ^{125}I-TT with 5 μg of nLTh-1 or 1.0 μg of nCT was nasally delivered to individual mice. The results are from five mice per group. Indicated are the means plus standard errors of the mean.

cules to the human nasal tract. Human studies involving nasal application of CT-B reported mild adverse effects, which resolved within 24 h (1, 39–41). The tolerated and effective dose was between 100 and 500 μg for nasally applied CT-B in a nasal spray/aerosol. At the lower end of an effective immunization range, i.e., 100 μg of CT-B, 7 out of 20 patients and all in the high range (the 1,000-μg group), experienced adverse effects. The symptoms consisted of profuse nasal secretions, itching, and violent sneezing, which resolved within 1 day (1). Although the use of a high-dose CT-B is different from our toxin-mediated antigen redirection, it could be argued that high doses of CT-B will accumulate in the olfactory neuroepithelium, as has been reported for mice (46), and consequently would lead to induction of proinflammatory cytokines throughout the nasal tract.

The results with nasally applied CT-B in humans are consistent with our observations that high levels of IL-6, and to a lesser extent IL-1β, are present in the nasal tract during the first 24 h after application. Interleukin 6 was expressed during the peak of TT protein redirection into the ON/E, and at 12 h was significantly higher in nCT-treated mice than in mice treated with mCT E112K. Furthermore, both enterotoxins induced significantly higher IL-6 levels in nasal washes than were seen in mice given TT only. IL-6 is a multifunctional cytokine that influences both innate immune reactions, such as inflammation (36) and acute-phase responses, and specific immunity, such as B-cell differentiation. IL-6 is produced by a variety of cells, including epithelial cells, macrophages, fibroblasts, and T cells. Native CT rapidly induces IL-6 secretion by the rat intestinal epithelial cell line IEC-6 (35). Whether IL-6 plays a

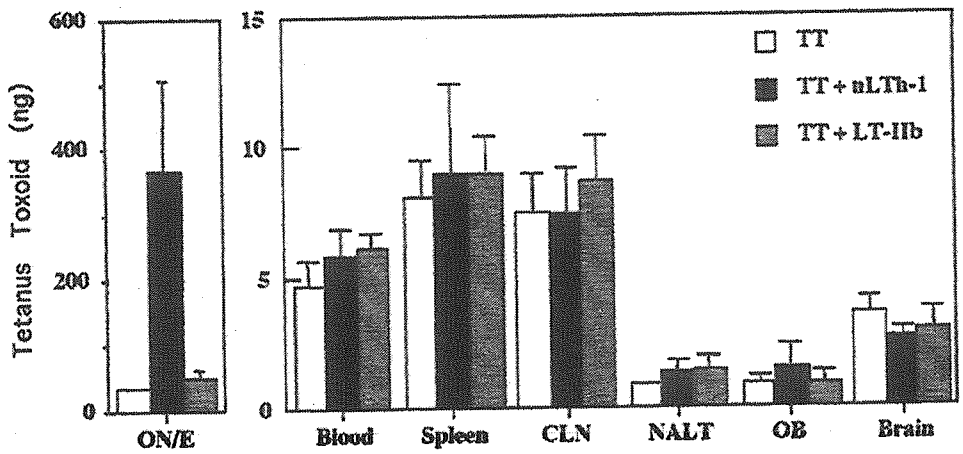

FIG. 6. Trafficking of ^{125}I-TT (20 μg) given nasally without or with nLTh-1 (5 μg) or LT-IIb (5 μg) as mucosal adjuvant. The uptake of ^{125}I-TT into the ON/E, OB, brain, NALT, CLNs, blood, and spleen is shown 12 h after nasal application. The results depicted are from five mice/group and are representative of three separate experiments. Indicated are the means plus standard errors of the mean.

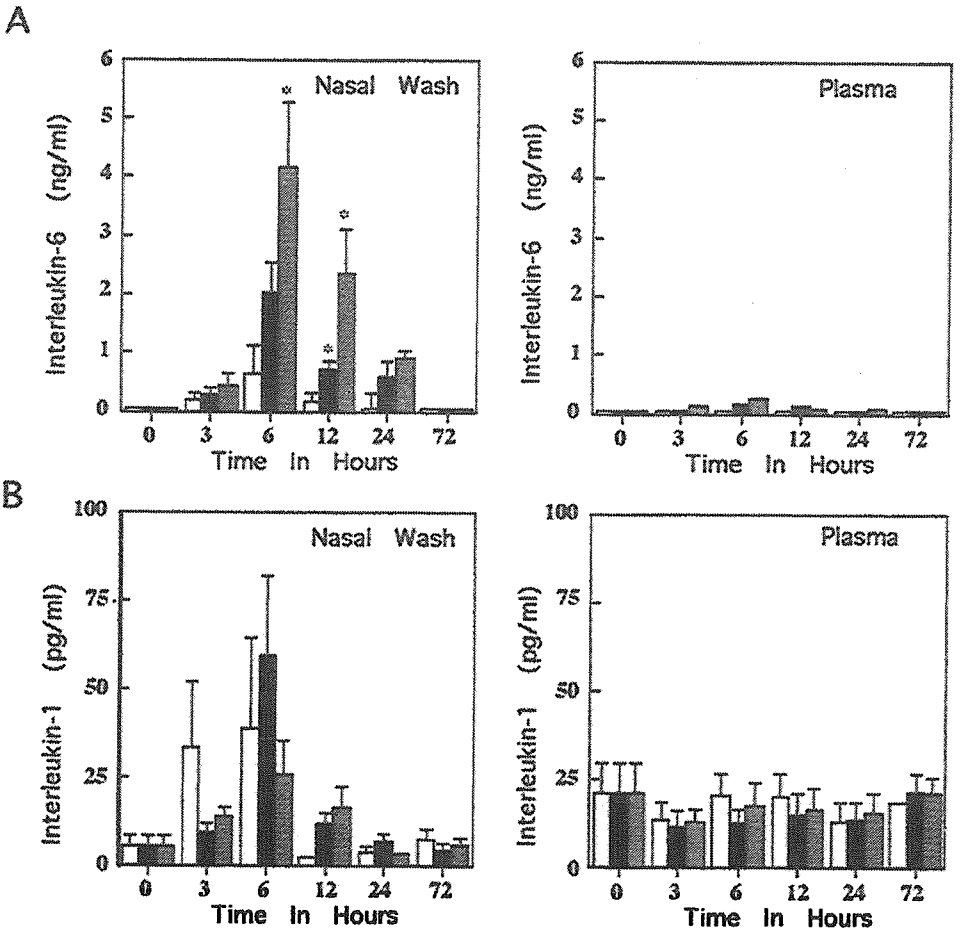

FIG. 7. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without mCT or nCT. Nasal washes were collected at 0, 3, 6, 12, and 24 h after nasal application of TT alone (open bars), TT and mCT (solid bars), or TT and nCT (hatched bars). The inflammatory cytokine levels for IL-6 (A), IL-1β (B), and TNF-α were determined by ELISA. No TNF-α was detected in the nasal washes. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β. The asterisks indicate significantly elevated cytokine levels ($P < 0.05$) when nCT or mCT with TT was compared with TT alone. The results are representative of two separate experiments.

role in antigen redirection remains to be determined; however, it is more likely that multiple factors contribute to antigen redirection into the ON/E. Specifically, neuropeptides could be major players in this process. For example, vasoactive intestinal peptide (VIP) plays an important role in fluid accumulation in the rat jejunum following stimulation with nCT or nLTh-1 (27). Furthermore, CT-B seems to specifically target VIP-containing neurons in the intestinal tract (15). These observations indicate that VIP could also be important for nasal reactogenicity and antigen redirection.

Human studies involving nasal application of CT-B have focused on the induced immune responses to CT-B rather than on its properties as a mucosal adjuvant (1, 39–41). However, the adjuvant properties of nLTh-1 were assessed in humans given two nasal applications a week apart with an aerosolized virosome-formulated influenza vaccine containing 1.0 μg or 2.0 μg of nLTh-1 for induction of influenza virus-specific immune responses (16, 17). The nLTh-1 functioned as a mucosal adjuvant in humans and induced influenza virus-specific immune responses; however, about 50% of the subjects experienced some type of local or systemic adverse reaction. These reactions included rhinorrhea, stuffiness, sneezing, and headaches, but most of them were mild and resolved within 48 h (16). Similar reactions have also been reported with nasal influenza virus vaccine given with 99.5 μg LT-B and 0.5 μg nLTh-1 (23). The results are consistent with our observations with nCT and nLTh-1 in that redirection of coadministered antigen into the ON/E and production of inflammatory cytokines resolved within 48 h, indicating that similar events could have taken place in humans.

Despite the similarities between mice and humans, the relative surfaces of the nasal tract that constitute the olfactory epithelium are quite different. In mice, approximately 45% of the nasal tract surface constitutes olfactory epithelium (19), while in humans it is an estimated 2.5 cm^2 which would translate to ~2 to 3% of the nasal surface (13, 22). Thus, in mice, nasal application is probably more likely to target olfactory neurons than in humans. Furthermore, the nasopharynx has a 90° angle in humans while there is only a 15° angle in mice. Due to the much larger volume of the human nasopharynx (20 ml) than the mouse nasopharynx (30 μl) and the larger angle, it is likely much harder to consistently target the olfactory

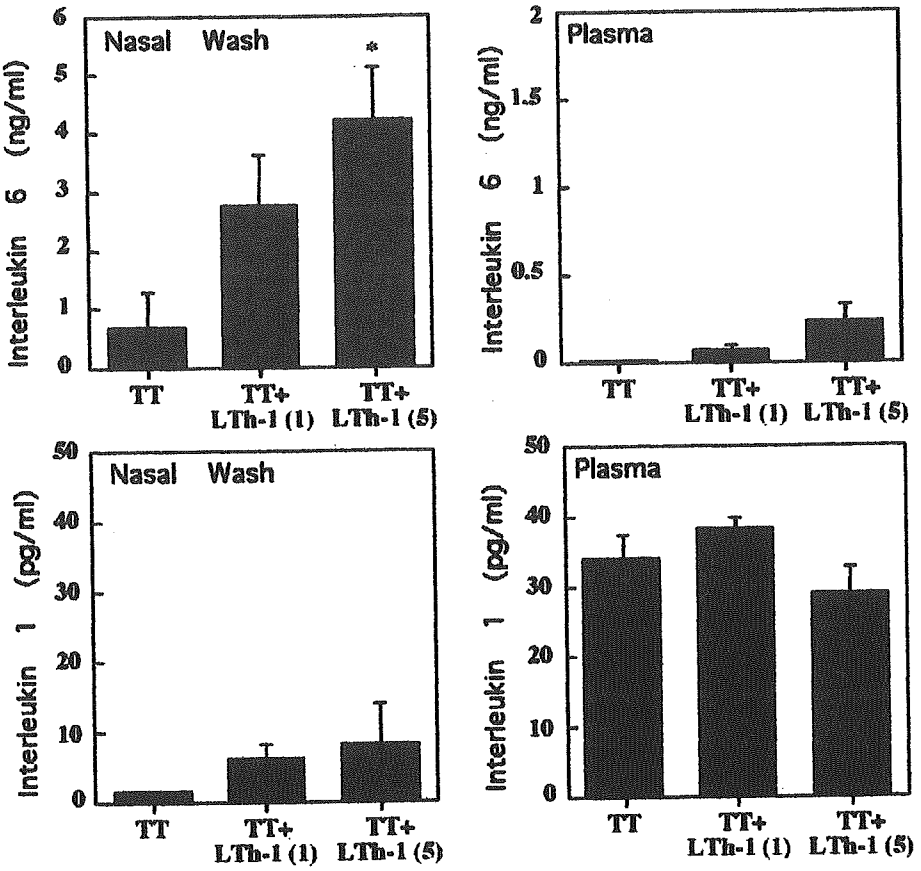

FIG. 8. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without LTh-1. Nasal washes and plasma were collected 12 h after nasal application of TT (10 μg) alone, TT and LTh-1 (1) (1.0 μg), or TT and LTh-1 (5) (5.0 μg). The levels of IL-6 and IL-1β in nasal washes and plasma were measured by ELISA. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β of five mice per group. The asterisk indicates significantly elevated cytokine levels ($P < 0.05$) when LTh-1 given with TT was compared with TT given alone. The results are representative of three separate experiments.

epithelium in humans using nasal drops, while in mice this would be very reproducible (13). It could be argued that because of the above-outlined reasons nasal sprays would more consistently target the olfactory epithelium in humans than nasal drops.

The observation that nCT significantly reduces TT accumulation in NALT 3 h after nasal application compared to TT alone or TT plus nLTh-1 (Fig. 4) is interesting from the perspective that exposure to a low dose of soluble protein is associated with induction of a Th2-type T helper cell response (6, 20, 37, 47). The induction of potent Th2-type helper activity specific for antigens codelivered with nCT (33, 50) or mCT (30, 51, 53, 54) and the induction of a mixed Th1/Th2 response to antigen coadministered with nLTh-1 (2, 7, 42), mLTh-1 (2, 7), or LTIIb (34) coincide with decreased antigen accumulation in NALT with a strong Th2 response but not with the mixed Th1/Th2 response (Table 1). For example, antigen accumulation in NALT is approximately sixfold lower with nCT than with nLTh-1. It will be interesting to see in future studies whether this altered antigen level will translate into an altered cytokine environment in the NALT for induction of a TT-specific immune response.

In summary, the redirection of a vaccine protein into the olfactory tissues by enterotoxin-based mucosal adjuvants following nasal administration is associated with reactogenicity in the nasal mucosa. The differential accumulation of TT protein in NALT when administered with nCT or nLTh-1 may have consequences for the induced TT-specific T helper cell responses. The parameters controlling antigen redirection into the ON/E include ADP-ribosyltransferase activity of the A subunit and GM1 ganglioside binding by the B subunit. Thus, redirection of vaccine antigen into the ON/E by enterotoxin-based mucosal adjuvants, such as nCT and nLTh-1, clearly requires both ADP-ribosyltransferase activity and targeting of GM1 gangliosides.

ACKNOWLEDGMENTS

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No cancer in cancers: Evolutionary trade-off between successful viviparity and tumor escape from the adaptive immune system

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Summary Some invertebrate species including the king crab and king squid enjoy relatively long lives of up to 20 years. Nevertheless, there are few reports of malignancies among invertebrate animals while there are many such reports in lower vertebrates such as in fishes, amphibians, and reptiles.

Viviparity is characteristic of most mammalian species, although it has been observed in both vertebrate and non-vertebrate species.

As adaptive immune responses evolved among the cartilaginous fishes by virtue of gene duplication, all viviparous vertebrates cope with specific immune responses to reject the fetal allograft.

The escape mechanisms employed by both human and animal malignancies share common properties, which are also employed by feto-placental units, such as the expression of non-classical major histocompatibility antigens (HLA-E, HLA-F, and HLA-G in humans), accumulation of regulatory T cells, Th2-directed immune responses, Fas/FasL- and/or PD-1/PD-L1-induced apoptosis, and the expression of indoleamine 2, 3 dioxygenase which starves the local tryptophan supply that is indispensable for an effective cytotoxic T cells response.

In humans, a single cancer cell requires 1–10 years to develop into a clinically remarkable tumor. For cancer cells, the genes encoding the immunoregulatory mechanisms employed by feto-placental units could be of value for escaping the host immune system.

Taken together, these observations support the author's hypothesis that the evolution of viviparity resulted in an evolutionary trade-off that may have increased susceptibility to malignancies.

Introduction

Viviparity is defined as the retention of developing embryos within the oviducts throughout development, a capacity which evolved more than 100 in different types of animals [1]. From an analysis of the fossil records and existing squamous reptiles, it was proposed that the evolutionary transition from oviparity to viviparity could occur quite rapidly, and that placentation evolved concurrently with viviparity [2].

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The animal kingdom is divided into two main categories: vertebrates and invertebrates. Invertebrates are considered to be all those animals that are not in the five main groups of vertebrates (Mammalia, Aves, Reptilia, Amphibia, and three classes of fish).

Some species of cartilaginous fish, amphibians, reptiles, and most mammals, except for platyps and echidna, are viviparous. Since jawed vertebrates have an adaptive immune system, it is crucial that these animals be able to protect their concepti from an alloimmune response.

Matsunaga proposed the hypothesis that the first adaptive immune system evolved as a gastrointestinal defense against microbial invasion as a result of increasing traumatization by injury and infection due to jaw development in host fish, which allowed them to capture and swallow foods (viz., other fish) [3]. He supported his hypothesis with the histological finding that the gut-associated lymphoid tissue (GALT) system is not developed in the hagfish or lamprey, which capture their food by a gentle sucking action (Fig. 1).

Neoplasms in lower animals

Neoplasias in invertebrates and evolutionary conservation of innate immune system

Proliferative lesions have long been recognized in invertebrate animals as well as in vertebrate animals. However, most of the reports on invertebrate neoplasms have focused on a small number of species including the fruit-flies (*Drosophia melanogaster*), cockroaches (*Periplanta* spp. and *Leucophaea maderae*), locusts (*Locustus migratoria*), beetles (*Melontha melontha*), and stick insects (*Carausius monosus*) [4]. Though the population and number of species of invertebrate animals are much greater than those of vertebrates, it is rare for biologists, fishermen, or cooks to encounter natural neoplasms in invertebrate animals, even if working regularly with arthropods and mollusks.

However, innate immune responses against malignancies have been reported in lower animals. Suzuki and Cooper reported on the spontaneous cytotoxicity of earthworm leucocytes (coelomo-

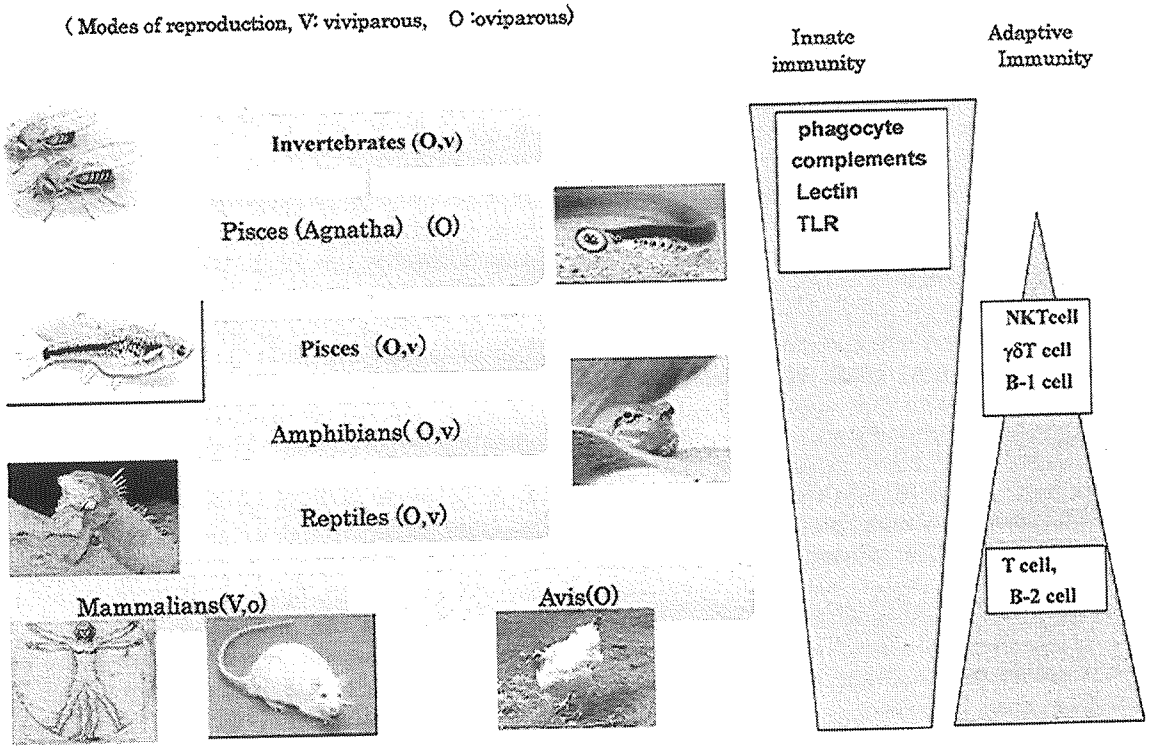

Figure 1 Modes of reproduction and evolution of immune system. Adaptive immune system mediated by genetically re-arranged immunglobulins and T cell receptors evolve at the level of jawed fishes. Though viviparity is not specific to mammalians, viviparous animals with adaptive immune system had to solve feto-placental surival against maternal immune recognition in utero.

cytes) against the NK sensitive human erythroleukemia cell line K562 [5]. Although the precise mechanism of cell recognition and contact-induced killing has not been clarified, their findings suggest the evolutionary conservation of an innate immune system that eliminates infected, mutated, and/or degenerated cells.

An innate immunity-dependent recognition of neoplasms has been reported in mammals. Complement-independent recognition and Toll-like receptor (TLR) mediated activation of host immune cells were observed against mutated virus-infected cells [6] without antigenic recognition. The human complement system protects an individual against substances of nonself origin, including xenografts and microbial pathogens. Human cells express the complement regulatory proteins, CD46 and CD55, thereby circumventing attack by C3, a major effector of complements. Seya's group reported that certain malignant cells, particularly those undergoing apoptotic stress, can activate the homologous complement, overcoming the regulatory actions of CD46 and/or CD55. They also identified a novel gene product, M161Ag, that converts human cells into targets for the homologous complement.

Members of the TLR family have been shown to be important in the activation of cells by a variety of microbial agents. Although TLRs have been considered to be important for defense against bacterial and fungal infections, recent studies have also proposed roles against viral infections including oncogenic viruses [7]. These findings suggest an evolutionary conservation of innate immunity in the surveillance of tumor cells by complement as well as TLRs (Fig. 1).

Neoplasias in fishes

Neoplastic disorders are common in fishes, which represent the largest group of vertebrates with over 20,000 documented species. Spontaneous, carcinogen/radiation-induced and viral-induced malignancies have been reported in both wild and domestic fishes [8]. On the other hand, neoplasms in several groups of fishes, such as the chondrichthyan (Class chondrichthyes) elasmobranches (sharks, skates and rays) and other primitive fishes including African lungfishes (Family protopteriae), bowfish (Family Amiidae), and chondrosteam fishes (Order Acipensiformes), are generally rare but have been reported. Grof attributed this rarity of tumors to their unique biology, such as the high ionic strength of elasmobranch tissues and antiangiogenic factors, and/or to a relative lack of observation and examination.

Neoplasms in amphibians and reptiles

Spontaneous neoplasia is not frequent in the three orders of Amphibia. However, tumors have been reported in most major organ systems with various etiologies, including viral infection, environmental contaminants, and genetic predisposition [9]. Anurans seem to have a greater frequency of spontaneous neoplasms than do urodeles and they respond to chemical carcinogens in a manner analogous to mammalian species [10].

Neoplasia is an important form of disease in saurians [11]. The organs most commonly affected by neoplastic disease are the hematopoeitic system, the hepatic system, the skin, and tumors of the musculoskeletal system. Hernandez-Divers et al. suggested that neoplasia should be considered as a significant differential diagnosis when presented with a lizard that has nonspecific clinical signs. Furthermore, recent years have seen an inexplicable increase in the frequency of an appalling disease in sea turtles: fibropapillomatosis, which is probably caused by a herpes virus and causes tumors to grow throughout the turtle's body [12]. Green turtle fibropapillomatosis is considered to be an increasingly significant threat to the survivability of this species.

Escape mechanisms employed by malignancies and concepti

In 1953, the famous British immunologist Sir Peter Medawar published an essay entitled "Some Immunological and Endocrinological Problems Raised by the Evolution of Viviparity in Vertebrates" [13], which became the most influential theory driving the development of the field of reproductive immunology over the next 50 years. He demonstrated the role of tissue antigens in the recognition and rejection of skin grafts between genetically differing individuals, and other researchers focused on the genetic basis of tumor transplant rejection in mice. These realizations led Medawar to recognize the truly paradoxical nature of the immunological relationship between the pregnant mother and her antigenically foreign fetus. He stated: "The immunological problem of pregnancy may be formulated thus: how does the pregnant mother contrive to nourish within itself, for many weeks or months, a foetus that is an antigenically foreign body?"

Having identified the problem, Medawar offered some possible solutions, proposing: "The reasons why the foetus does not habitually provoke an immunological reaction from its mother may be classified under three headings: (a) the anatomical separation of foetus from mother; (b) the antigenic

immaturity of the foetus; and (c) the immunological indolence or inertness of the mother".

Later, he developed an interest in the expression of stage-specific fetal and onco-fetal antigens, in the context of their potential for the generation of anti-cancer vaccines [14].

A crucial feature of the maternal–fetal relationship is that the two circulatory systems remain almost completely separate throughout the period of gestation. The original concept of "the embryo, quâ tissue homograft", is not therefore, in the strictest sense, appropriate. It is at the level of the placenta, and the fetal membranes, that tissue contact between the fetal graft and the maternal host is made. The placenta itself is provided with a continuous and unbroken outer barrier layer of trophoblastic tissue, which exists together with a variety of other biological forms of trophoblast situated at different anatomical locations within the pregnant uterus. The feto–maternal interface at the non-placental regions of the implantation site consists of the outermost of one of the extra-embryonic fetal membranes, which in man is trophoblastic in nature but in other species, notably laboratory rodents, is non-trophoblastic. The true fetal allograft of pregnancy is, therefore, the trophoblast and extra-embryonic membrane(s), both having direct cellular contact with the maternal uterine environment [15].

Recent advances in reproductive immunology have revealed more the detailed mechanisms involved in the maternal–fetal relationship and have shed light on why feto-placental units are not susceptible to attack by the maternal immune system (Fig. 2). Surprisingly, most escape mechanisms employed by mammalian concepti have shown striking similarities to those employed by human malignancies, as well as with those observed in experimental animals.

Burnet and Thomas proposed an immuno-surveillance hypothesis, which holds that the immune system can recognize and destroy nascent transformed cells. However, this hypothesis was soon abandoned due to the absence of strong experimental evidence supporting the concept. Recently, there has been an accumulation of experimental data supporting the old hypothesis of cancer immunosurveillance and indicating that it may function as a component of a more general process of cancer immunoediting. This process is responsible for eliminating tumors, sculpting the immunogenic phenotypes of tumors that eventually form in immunocompetent hosts, and facilitating tumor escape from immune destruction [16].

Non-classical HLA HLA-G and HLA-E

Both concepti and several types of malignancies are known to escape from the cytotoxic T cell (CTL) response and NK cell-mediated cell lysis by monomorphic (or oligomorphic) major histocompatibility complex class I molecules. Engagement by the T cell receptor (TCR) of a complex between the peptide and MHC (peptide-MHC) on antigen-presenting cells (APCs) is required for the development of T cells in the thymus, their survival as naïve cells in the periphery, and their activation and effector functions. In humans, there are six major groups of HLA (human leukocyte antigens): A, B, C, D, Dr, and Dq. They are categorized as classical HLA and they act as antigens when transplanted to recipients.

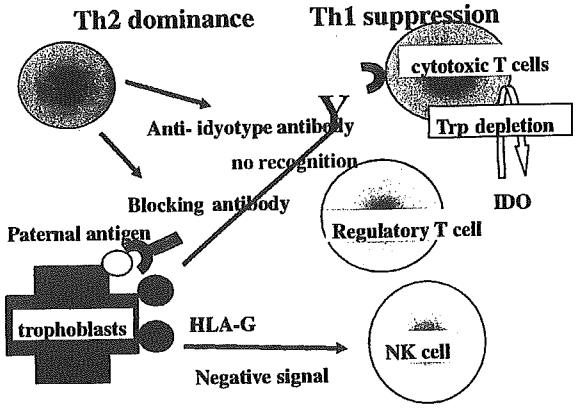

Figure 2 Immune responses to allogenic fetus.The mammalian fetus, except in instance in which the mother and the father are syngenic, will express paternally inherited antigen that are allogenic to mother. Protection of semi-allogenic fetus against maternal immune system involves several mechanisms. These mechanisms share common properties employed by malignancies.

HLA-G is a non-classical MHC-I molecule that is primarily expressed at the fetal–maternal interface, where it is thought to play a role in protecting the fetus from the maternal immune response. HLA-G binds a limited repertoire of peptides and interacts with the inhibitory leukocyte Ig-like receptors,LIR-1 and LIR-2, and possibly with certain natural killer cell receptors. The expression of the non-classical HLA-G molecule on various cancer cell lines and clinical samples including renal clear cell carcinoma [17], ovarian carcinoma [18], melanoma [19], and breast carcinoma [20] has been reported. The expression of HLA-G in cancer represents a strategy employed by tumors to avoid immune destruction. Hansel et al. reported recently that the up-regulation of HLA-G is associated with a neoplastic progression of pre-malignant and malignant lesions of colorectal epithelial tumors [21]. On the other hand, Dutta et al. have reported an absence of HLA-G but frequent expression of another non-classical antigen, HLA-E mRNA, in primary gastric carcinomas [22]. Expression of these non-classical HLAs in cancer represents a strategy employed by tumors to avoid immune destruction. Indeed, this non-classical HLA class I molecule suppresses various immune cell functions through binding to inhibitory receptors.

It is believed that MHC-deficient tumor clones can escape T-cell immune responses, but are in theory more susceptible to NK-cell-mediated lysis. However, if they express non-classical HLA class I molecules, they escape via NK inhibitory receptors [23]. The up-regulation of HLA-G gene transcription by tumor environmental factors such as cytokines, stress, and agents used in chemotherapy such as demethylating molecules has been reported as has the subsequent evasion of malignant cells from the antitumor immune response.

Surprisingly, increased expression of HLA-G was observed in endomyocardial biopsy specimens obtained from heart transplants, without chronic rejection [24]. Taken together,these findings suggest that HLA-G may be a master-key molecule for evading allograft rejection that has been gifted by evolution.

Indoleamine 2,3-dioxygenase (IDO)

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that degrades the essential amino acid tryptophan. In murine experimental systems, the expression of IDO correlated with reduced T cell-mediated responses in autoimmune diseases, cancer, organ and tissue transplant rejection, and pregnancy [25]. Pregnant mice treated with IDO inhibitors experienced fetal resorption associated with extensive inflammation complement deposition and hemorrhagic necrosis at the maternal–fetal interfaces. The fetal allograft rejection was completely allo recognizing T cell-dependent because it was not observed in mice carrying syngeneic fetuses nor in T cell-deficient mice. These data show that IDO activity protects the fetus by suppressing T cell-driven local inflammatory responses to fetal alloantigens. IDO is expressed in dendritic cells as well as macrophages. In human placental tissue, Honig et al. localized the expression of IDO in syncytiotrophoblasts and endothelial cells as well as extravillous trophoblasts (EVT) by immunohistochemical techniques. They reported that EVT is the main source of IDO with blocking experiments [26].

In cancer tissues, increased expression of IDO has been reported in plasmocytoid dendritic cells in tumor drainage lymphocytes [27] as well as in carcinoma cells themselves such as in cervical carcinoma [28], esophageal carcinoma [29], and estrogen receptor negative breast cancer cell lines [30]. The expression of IDO has recently been reported to be controlled by a transcription factor, Bin1, which is attenuated in many human malignancies. Further, mouse knockout studies have shown that Bin1 loss elevates the STAT1- and NF-kappaB-dependent expression of IDO, driving the escape of oncogenically transformed cells from T cell-dependent antitumor immunity [31].

Release of immunosuppressive cytokines

Both malignancies and concepti secrete regulatory molecules including cytokines and prostanoids to evade both local and systemic immune responses. Cytokines are low molecular weight proteins that use their ability to act as intercellular communicators to regulate the immune response. A variety of cell types, principally T-helper lymphocytes and macrophages, can secrete cytokines in response to various stimuli. The functions that cytokines induce can both turn on and turn off particular immune responses.

Many types of malignancies have taken advantage of the regulatory role of cytokines to down-regulate appropriate immune responses targeted at destroying them. Cancers secreting immunosuppressive cytokines have been reported to be more aggressive and are associated with higher metastatic rates, and shorter survival periods of the hosts. For example, interleukin-6 (IL-6), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF), Granulocyte colony stimulation factor (G-CSF), and transforming growth factor-beta (TGF-β) secreted by cancers often induce generalized

and specific inhibition of immune responses. We have reported immuno-regulatory roles of placenta-derived G-CSF in pregnant subjects as well as its suppression on autologous tumor killing activity in patients with ovarian carcinoma [32,33]. Of particular importance is the finding that most lymphocytes, including NK cells, NKT cells, and alpha–beta or gamma–delta T cells accumulating at tumor sites [34] produce IL-4, IL-10, and TGF-β and possibly inhibit CTLs and T helper 1 (Th1) cell responses, as observed in decidual intraepithelial lymphocytes. Gamma–delta T cells with regulatory roles were independently discovered by the author and Seo in deciduas and tumor tissues [35–37].

Secretion of prostaglandins

Prostaglandins secreted from cancer tissue may affect the host immune response as observed in decidual placental units. Prostaglandins affect cell differentiation, proliferation, and apoptosis, as well as target cell interaction. In human tissues, monocytes and macrophages are the cells responsible for prostaglandin production. Monocytes and related cells play a key role in regulating the interleukin cascade leading to T cell proliferation and finally to the immune response. They can either amplify the response by producing interleukin 1 or shut it down mainly by releasing prostaglandin E2. In vitro, prostaglandins have been shown to inhibit lymphocyte mitogenesis, cytolysis, and antibody production. In cancer tissues, increased expression of cyclooxygenase-2 (COX-2) has been reported in various human neoplasms including malignancies of the gastrointestinal tract and reproductive organs,in brain tumors, and in cancers of cancers of the head and neck. Increased production of prostanoids and subsequent local or systemic immune suppression are attributed to poor prognosis in patients with COX-2 positive tumors.

A hypothesis presented by Tawfik explains PGE2 as a primary suppressor molecule in decidual tissue. They observed a high concentration of PGE2 and PGF2alpha in cultured murine decidual cells, and transferable immuno-suppressive activity with reversal by indomethacin, an inhibitor of prostaglandin synthesis [38]. In humans, Kvirkvelia et al. observed selective expression of COX-2 in the syncytiotrophoblast of the chorionic villi and demonstrated an immunosuppressive effect of PGE2 on CTLL-2 cells via the EP4 receptor using highly selective antagonists [39]. Recently, trophoblast, uterine epithelium, and endometrial glands have all been shown to express haematopoietic-type prostaglandin (PG) D_2 synthase (hPGDS) and to recruit Th2 cells and Tc2 cells from peripheral circulation to the materno-fetal interface (implantation site) by chemoattraction mediated by PGD_2 and its receptor, CRTH2 [40].

Lack and aberrant expression of co-stimulation molecules

The presence of inadequate, inappropriate, or inhibitory T cell co-stimulatory pathway signaling has been shown to restrict a host's ability to generate productive immune responses against carcinomas and concepti. The expression of B7 on the surface of a cell is the co-stimulatory signal necessary to allow for the cytolytic CD8+ T cell attack on the targets. The co-stimulation results from an interaction of the CD28 molecule on the T cell surface with its ligand, B7, on the surface of an antigen-presenting cell (APC). The B7 display renders target cells capable of effective antigen presentation, leading to their eventual eradication. Related to this, the co stimulatory ligand, B7-H1 (PD-L1), has recently been implicated as a negative regulator of antitumoral T cell-mediated immunity. The expression of B7-H1 is normally restricted to macrophage-lineage cells, providing a potential co-stimulatory signal source for the regulation of T cell activation. In contrast, an aberrant expression of B7-H1 by tumor cells has been implicated in the impairment of T cell function and survival, resulting in defective host antitumoral immunity. B7-H1 expressed by tumor cells has been shown to enhance apoptosis of activated tumor-specific T cells in vitro [41]. Consistent with these observations, an in vivo monoclonal antibody blockade of B7-H1 has been shown to potentiate antitumoral responses in several murine cancer models.

The expression of B7-H1 has been described in a number of human malignancies including non-small cell lung carcinoma [42], ovarian carcinoma, and renal cell carcinoma [43].

In placental tissues, the mRNA for B7-H1 (PD-L1) and B7-DC (PD-L2) was reported to be highly expressed throughout gestation [44,45]. Petroff et al. localized an abundant expression of B7-H1 in cytototrophoblasts and syncytiotrophoblasts, as well as in extravillous cytotrophoblasts, all of which were juxtaposed to maternal blood and decidual tissue. They proposed the existence of a mechanism for active suppression of the maternal immune system that still maintains the ability to protect against foreign pathogens [46].

A second receptor for B7 on Tcells is cytotoxic lymphocyte antigen-4 (CTLA-4), the engagement of which provides an inhibitory signal that is imperative for the negative regulation of the immune

system [47] Constitutive expression of CTLA-4 mRNA and protein was reported in fetal tissues at the maternal–fetal interface throughout gestation [48], as well as in various cancer tissues [49].

Th2 predominance in pregnant women and cancer patients

Helper T cell responses differ between the two subpopulations, Th1 and Th2, according to differences in their cytokine expression profiles. IFN-γ, secreted from Th1 cells, is known to induce the differentiation of Th0 cells to Th1 cells and to inhibit the proliferation of Th2 cells. IL-4 and IL-10, secreted from Th2 cells, are known to induce the differentiation of Th0 to Th2 cells and to inhibit the function of Th1 cells [50]. It is widely believed that a Th2-dominant cytokine environment is necessary for successful pregnancy, while the actions of Th1 cytokines are thought to be detrimental to the fetus [51,52].

In cancer bearing patients, Th1 immunity plays a key role in the host defense against tumors, while a Th2-dominant environment has been shown to favor tumors [53–56].

The regulation of Th1/Th2 responses has been shown to be critically important for antitumor immune responses, such as the inhibition of tumor growth and metastasis, and increased survival rates in experimental animals. In humans, it was reported that the T cell responses shifted from Th1- to Th2-dominant status depending on the malignancy stage [57].

CD4+ CD25+ T regulatory cells (Treg)

CD4+ CD25+ T regulatory cells (Treg) are thought to be a functionally unique population of T-cells. They suppress antigen-specific immune responses and are important for allograft tolerance and suppression of autoimmune responses as well as for successful pregnancy both in mice [58] and humans [59,60]. These data support the concept that normal pregnancy is associated with an elevation in the number of Treg cells which may be important in maintaining materno-fetal tolerance.

In cancer immunology, experimental animal models and patients with various neoplasms have shown an increase in circulating Treg cells accompanied by their increased involvement in down-regulation of effector functions against tumors, resulting in T-cell dysfunction in cancer-bearing hosts [61–66]. Of particular interest, is the finding that the population of Tregs in tumor-infiltrating lymphocytes (TILs) of patients with advanced gastric cancer with a poor prognosis was significantly higher than that of TILs in patients with early gastric cancer.

The increase of Treg cells during pregnancy is partially attributed to estrogen [67]. The peripheral blood Treg population peaks during the second trimester and then declines in the postpartum period.Possible roles of placenta-derived endocrine factors or chemokine/cytokine control on local accumulation have been suggested but have not been adequately documented. Treg cells specifically express the chemokine receptors CCR4 and CCR8 and respond to the chemokines macrophage-derived chemokine (MDC/CCL22), thymus and activation-regulated chemokine (TARC/CCL17), I-309/CCL1, and the virokine vMIP-I, which are agonistic ligands of these receptors. As reported by the authors and other researchers, TARC, MDC, and other fraktalkines are produced in the villous and extra-villous trophoblasts in human placentae [68]. These chemokines, since they are produced by various human malignancies, are likely candidates to accumulate Treg in cancer tissues.

Trade-off in evolution and viviparity

The concept of a trade-off represents a key paradigm in evolutionary medicine. Over the last few decades there has been considerable effort to introduce an evolutionary perspective to biomedical research in the context of examinations, encouraging both researchers and clinicians to ask questions pertinent to the origin, and not simply the management, of a disease [69,70].

The concept of trade-off is common in economics, denoting choices made to accept less of one thing in order to acquire more of another. For example, when one is allocating (limited) funds, the trade-off usually involves reduced spending for some purposes to allow spending for other, perhaps more urgent purposes [71]. This concept can be readily extended beyond decisions involving money to human behaviors and non-human events. During evolution based on Darwinian selection, trade-offs were important drivers of biological events such as host–parasite relationships between humans and microorganisms [72,73], mate-choice decisions by female crickets [74], and the coexistence of specialists and generalists on an ecological timescale [75].

As individuals are always at risk of death, selection favors early reproductive opportunities over the potential for later ones. Accordingly, selection is never more efficient than at the age of commencement of reproduction, and this efficiency declines thereafter. Thus, traits that have beneficial effects

in early life will tend to spread, even if inseparately coupled to deleterious late effects [76]. Crespi and Semeniuk hypothesized that parent–offspring conflict over the degree of maternal investment has been one of the main selective factors in the evolution of the vertebrate reproductive mode [77]. It is proposed that in all organisms where parents and their offspring are not genetically identical, conflicts of interest will arise between them over the level of parental investment [78]. Their hypothesis is supported by several lines of evidence: the high number of independent origins of viviparity, matrotrophy (direct maternal–fetal nutrient transfer), and hemochorial placentation (direct fetal access to the maternal bloodstream); the extreme diversity in physiological and morphological aspects of viviparity and placentation, which usually cannot be ascribed adaptive significance in terms of ecological factors; and the divergent and convergent patterns in the diversification of placental structure, function, and developmental genetics. Furthermore, embryos and fetuses, like neoplasms, actively manipulate their interaction with the mother, thereby garnishing increased maternal resources. For the mothers, each of her genes has an equal probability of being present in each offspring, so her best strategy involves allocating nutrients to each offspring over her lifespan to maximize the aggregate reproductive success of her descendants. For offspring, the effects of genes are expected to be more selfish such that offspring are selected to seek greater investment from the mothers than she is selected to provide. Thus, intra-genomic conflict may occur when some elements in the genome – the so-called selfish – produce effects that enhance their own probability of replication or transmission at the expense of other elements within the same genome. For example, both protooncogenes and tumor suppressor genes have played crucial roles in cellular functions across hundreds of millions of years of evolution, and tumor suppressor genes have been shown to carry out policing functions in invertebrates as well as in vertebrates [79]. Summers et al. proposed that both parent–offspring and intra-genomic conflicts apparently favor the evolution of alleles that promote cancer [80].

Conclusions

The principal functions of the immune system in long-lived metazoans are supposed to be the defense against microbial pathogens and the surveillance and clearance of aberrant components of self. Viviparity requires immune suppression in pregnant females to protect their concepti from immunological attacks. For transformed cells, genes employed by feto-placental units allow them to escape detection by the host immune system. Given these findings and hypotheses, the author propose that the evolution of viviparity resulted from a trade-off that increased susceptibility to malignancies in exchange for survival of the feto-plancental unit against the maternal immune system. This hypothesis would also explain the paucity of malignancies among invertebrate animals. Our ancestors may have opted for the big-ticket purchase of successful viviparity while paying for it in part with a somewhat compromised immune defense system.

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Rembrandt's Bathsheba, possible lactation mastitis following unsuccessful pregnancy

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Summary Deformity of the left breast and axilla observed in Rembrandt's famous painting "Bathsheba at her toilet" (1654, Louvre Paris) has been discussed by several researchers. Proposed diagnoses were breast cancer and abscess due to tuberculosis. The present article reviews previous articles written concerning the left breast abnormalities of Bathsheba and carefully examines other works of Rembrandt modeled by Hendrickje and painted around 1654. Previous diagnosis of breast cancer and tuberculous mastitis is less probable. Because Hendrickje survived for more then 9 years after the painting and in other works modeled by Hendrickje shows no signs of cachexia or permanent changes in the left breast. The most likely diagnosis of the left breast deformity of Bathsheba is a sequela of lactation mastitis abscess following miscarriage or premature childbirth without breast feeding.

A case study and proposed diagnoses

Rembrandt van Rijn (1606–1669) born in Leiden was one of the most famous and leading representatives of the Dutch School of painting and was especially talented in the use of light and shadow [1]. He left correct paintings of medical scenes known as "Anatomy lesson by Dr. Nicholaes Tulp" (1632, The Hague, Mauritshuis museum) and "The anatomy lesson of Dr. Joan Dejiman" (1656, Amsterdam Historisch Museum).

Other than medical paintings, he recorded clinical findings of basal cell carcinoma in "Man in oriental costume" (1632, New York metropolitan Museum) [2], signs of ageing including brow and eyelid ptosis, rosacea and temporal arteritis in his self-portrait (1659, National Gallery of Art, Washington, DC) [3].

In 1983 Braithwaiste and Shugg suggested that Rembrandt's famous painting of "Bathsheba at her toilet" (1654, Louvre Paris) showed clinical signs of advanced left breast carcinoma based upon skin discolouration, distortion, axillary fullness and peau d'orange appearance [4]. The breast cancer hypothesis was presented independently by Dymarskii in 1984 [5].

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