Cholera toxin B stimulates systemic neutralizing antibodies after intranasal co-immunization with measles virus

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An efficient mucosal vaccination has a number of obvious advantages over invasive routes of immunization. The immune response to measles virus (MV) was investigated after intranasal and intragastric co-immunization of mice with cholera toxin B (CTB) as an adjuvant. High titres of virus-specific IgG antibodies and a transient IgA response were detected in the sera after intranasal but not after intragastric immunization when CTB was used. In the presence of CTB, higher titres were reached with less antigen and fewer intranasal boosts. Neutralizing antibodies were found in all animals only after co-immunization with MV and CTB. In the nasal wash and the saliva, IgG and IgA titres were significant only in the MV plus CTB groups; IgG levels were comparable to those found after intraperitoneal (i.p.) immunization with complete Freund's adjuvant. Specific IgA was detected in the mucosal fluids only after intranasal immunization with MV plus CTB but not after i.p. or intragastric immunization. The antibody response consisted of 99% IgG1 after MV immunization. In the CTB groups 10% IgG2b and 1% IgG2a were detected in addition to the predominant IgG1 antibodies.

Introduction

Efficient mucosal vaccines have a number of advantages over invasive vaccinations. The most obvious include simplicity, safety and cost-effectiveness. Mucosal vaccines target antigens directly to the lymphoid tissue associated with the mucosal surfaces of the digestive and respiratory tract, which represent the largest tissue masses of the immune system (Lamm, 1976; Weisz-Carrington et al., 1979).

Many antigens are poorly immunogenic after mucosal administration, which has limited their use as local vaccines. In order to induce a strong mucosal immunity, potent and safe adjuvants are normally required. Cholera toxin (CT) has been found to be a mucosal immunogen and a potent mucosal adjuvant, capable of abrogating oral tolerance to unrelated antigens (Elson & Ealding, 1984; Lycke & Holmgren, 1986). The enterotoxigenic activity responsible for diarrhoea in cholera is mainly confined to toxin subunit A.

In conjunction with different antigens and/or routes of immunization the subunit B (CTB) of the cholera enterotoxin was found to be sufficient for a strong adjuvant effect (Chen & Strober, 1990; McKenzie & Halsey, 1984; Tamura et al., 1992). CTB is non-toxic and has been used extensively in humans as a component of a cholera vaccine (Clemens et al., 1990). Considerable experience has been accumulated from immunizations with CTB combined with protein antigens (McKenzie & Halsey, 1984; Menge et al., 1993; Dertzbaugh et al., 1990) including viral proteins (Tamura et al., 1992; Lehner et al., 1994; Gonzales et al., 1993). These were either mixed with CTB (Tamura et al., 1992), conjugated with cross-linking agents (McKenzie & Halsey, 1984; Menge et al., 1993) or genetically fused (Dertzbaugh et al., 1990) to the B subunit. Despite encouraging results, much less is known about the adjuvant effect of CTB in combination with viral particles (Mbawuiki & Wyde, 1993; Chen & Strober, 1990; Israel et al., 1992; Liang et al., 1988). In most studies antiviral immune responses were enhanced when viral proteins were combined with CTB or the holotoxin; e.g. co-immunization of influenza haemagglutinin and CTB protected mice against lethal virus challenge (Tamura et al., 1992). In a number of systems including gut immunization against viruses the holotoxin was more efficient as an adjuvant than its subunit alone (Czerkinsky et al., 1991; Liang et al., 1988; Lycke & Holmgren, 1986; Lycke et al., 1989). For

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instance, cholera toxin, when used as an adjuvant, stimulated antibody responses to UV-inactivated respiratory syncytial virus (RSV) that were similar in quantity and distribution to those of live RSV infection (Reuman et al., 1991a).

The combination of live and in particular of non-replicating viral vaccines with enterotoxicogenic adjuvants appears to be a valid strategy for mucosal vaccination. Non-replicating viral vaccines are currently being developed against a variety of viruses including the measles virus (MV; Taylor et al., 1992). We have extended the above observations with CTB, to investigate the systemic and local antibody response in the mouse after mucosal immunization with MV and enterotoxins. Systemic neutralizing antibodies are known to protect against measles (Black, 1989). It is of interest to note that passively acquired IgG antibodies interfere less with viral vaccines administered mucosally than with those administered parenterally (Murphy et al., 1989; Jayashree et al., 1988; Kimman & Westenbrink, 1990) and that mucosal immunization can be successful despite the presence of maternal antibodies (Sabin et al., 1983). In addition to the systemic immunity, a local immune response can be expected to have collateral benefits since the oropharyngeal cavity represents the main portal of entry for the MV and mucosal tissues are main sites of MV-related pathology and complications such as pharyngitis, conjunctivitis, measles-associated diarrhoea, pneumonia and (obstructive) laryngitis (Ross et al., 1992). Moreover, breast-fed infants could potentially benefit from boosted virus-specific antibodies.

Our main findings in this study were that CTB improved the immune response to the MV after intranasal immunization by boosting mucosal antibodies and inducing systemic virus-neutralizing antibodies, whereas CTB or the heat-labile E. coli enterotoxin were unable to induce a significant immune response after intragastric immunization.

Methods

Enterotoxin. CTB from Vibrio cholerae (34-8 toxoid units/μg protein; permeability factor activity < 0.02%) was purchased from Sigma. Residual toxic activity was measured by the adenylate cyclase assay as described (Gill & Rappaport, 1979). The CTB preparation used in this study (batch 051H0852) was 99.99% choleragenid by weight. The combination of live and in particular of non-replicating viral vaccines with enterotoxicogenic adjuvants appears to be a valid strategy for mucosal vaccination. Non-replicating viral vaccines are currently being developed against a variety of viruses including the measles virus (MV; Taylor et al., 1992). We have extended the above observations with CTB, to investigate the systemic and local antibody response in the mouse after mucosal immunization with MV and enterotoxins. Systemic neutralizing antibodies are known to protect against measles (Black, 1989). It is of interest to note that passively acquired IgG antibodies interfere less with viral vaccines administered mucosally than with those administered parenterally (Murphy et al., 1989; Jayashree et al., 1988; Kimman & Westenbrink, 1990) and that mucosal immunization can be successful despite the presence of maternal antibodies (Sabin et al., 1983). In addition to the systemic immunity, a local immune response can be expected to have collateral benefits since the oropharyngeal cavity represents the main portal of entry for the MV and mucosal tissues are main sites of MV-related pathology and complications such as pharyngitis, conjunctivitis, measles-associated diarrhoea, pneumonia and (obstructive) laryngitis (Ross et al., 1992). Moreover, breast-fed infants could potentially benefit from boosted virus-specific antibodies.

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Measles virus preparation. Vero cells were grown to subconfluency at 37 °C in RPMI 1640 (Gibco) supplemented with penicillin (100 IU/ml), streptomycin (1 μg/ml), 5% fetal calf serum (or alternatively 2% Ultrosor; Gibco), 2 mM-glutamine, 50 μM-mercaptoethanol and infected with MV (Edmonston strain; ATCC VR-24) at an m.o.i. of 0.3. After infection the culture was kept at 31 °C. On day 5 post-infection (p.i.) the supernatant was harvested. Cell debris was removed by centrifugation at 1500 g and filtration through a 0.65 μm filter. The MV was concentrated and purified by ultracentrifugation on a discontinuous 30/70 % sucrose density gradient. The 30/70 % interphase was washed in TNE buffer (50 mM-Tris–HCl, 100 mM-NaCl, 1 mM-EDTA) and ultracentrifuged onto a 70 % sucrose cushion. The final preparation contained 1.3 × 10^9 haemagglutinating units (HAU).

Animals. Male and female specific-pathogen-free (SPF-5) BALB/c mice were obtained from the Laboratory Animal Center of the Department of Immunology, Erasmus University, Rotterdam (The Netherlands). In our laboratory the animals were kept in a positive pressure laminar flow cabinet (UWO, Zevenaar, Holland) and used for immunization at 10 to 20 weeks of age.

Immunization protocols. Groups of four BALB/c mice were immunized intranasally with 300 to 2400 HAU of MV mixed in PBS with or without 10 μg of CTB. Animals immunized with 10 μg CTB or PBS alone served as negative controls. Prior to the first immunization, control sera were obtained from each mouse. For immunization the mice were held supine and induced to breathe in 5 μl (through each nostril) of a PBS-antigen solution which was carefully dispensed from a micropipette in a non-traumatic fashion. Intranasal immunization and intranasal boosting on days 14, 28 and 92 were used as the standard protocol. Oral immunization was performed according to the same standard schedule using a straight intragastric feeding needle to dispense 250 μl of antigen preparation in 0.5 M-bicarbonate buffer. Intraperitoneal immunization was done with 300 or 1200 HAU of MV emulsified in equal volumes (250 μl) of PBS and complete/ incomplete Freund’s adjuvant (CFA) following the standard schedule.

Sample collection. Serum was obtained by retro-orbital bleeding on day 0, 14, 28 (before the second boost), 35 and/or 49 and 97. For harvesting saliva the animals were injected intraperitoneally with 0.5 ml of 0.002 % pilocarpine (Sigma). The saliva was collected using a dull 20 gauge feeding needle connected to a syringe. All animals were sacrificed on day 97 (5 days after the third boost). Bronchoalveolar lavages were performed on the dead animal by intubating the trachea with a needle and flushing the lungs three times with the same volume of Hank’s balanced salt solution (750 ml) containing penicillin and streptomycin. About 400 ml fluid was recovered. The nasal wash was performed by intubating the nasopharynx of the dead animal with a 100 μl Eppendorf tip and flushing the nasal cavity once with 500 μl PBS supplemented with antibiotics. The gut wash was done by flushing 12 to 15 cm of jejunum with 1 ml PBS containing a cocktail of protease inhibitors (0.25 mM-PMSF, 0.02 % EDTA, 0.05 % sodium azide, 0.01 % soybean trypsin inhibitor). All organ washes were centrifuged to remove cells and debris and stored at −20 °C.

ELISA. Anti-MV antibody levels were measured using a commercial ELISA based on MV-infected diploid human cells (Enzygnost; gift from Dr Giesendorf, Behringwerke Marburg, Germany) following the supplier’s instructions. In short, 100 μl of diluted fluid (dilution buffer TBS containing 0.1 % Tween and 1 % BSA) was incubated for 30 min and washed with Tris-buffered saline (TBS, pH 8) containing 1 % Tween. Affinity purified alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) from Sigma or goat anti-mouse IgA (Southern Biotechnology Associates) were used for detection. Microtitre plates coated with MV-free cells served as negative antigen controls. Endpoint titres were determined by testing serial two-fold dilutions. The endpoint titre was defined as the highest dilution which gave rise to a difference of 0.05 A405 units between the MV-coated wells and the MV-free antigen control wells. Intermediate values were interpolated. Serum endpoint titres below 1:100 for IgG and below 1:25 for IgA were considered negative. In the nasal, lung and gut washes titres below 1:10, 1:10 and 1:5, respectively were considered negative.
Fig. 1. Time profile of MV-specific endpoint titres of IgG (a) and IgA (b) after intranasal immunization with 600 HAU of MV in the presence or absence of CTB. Animals were immunized on day 0, 14, 28 and 92. Sera were harvested on day 14, 28 (before boosting), 49 and 97. The bars represent reciprocal endpoint titres from individual mice. At each time point a group of four mice immunized with MV or MV/CTB were bled. The data from each group of four mice are separated by a tick mark. The IgG scale is truncated at a titre of 1:100000 (MV/CTB, day 97, 1:115000). Lowest titres tested were 1:100 for IgG or 1:25 for IgA. Sera that tested negative for IgG were attributed a titre of 1:100. P values compare mean log~ transformations of titres of the groups co-immunized with MV/CTB with the corresponding groups immunized with MV alone. IgG: day 14, P < 0.05; day 28, P < 0.04; day 49, P < 0.05; day 97, P < 0.004. IgA: day 14, not significant (NS); day 28, P < 0.00004; day 35, P < 0.00006; day 49, P < 0.001; day 97, NS. Day 97, MV versus negative control: P < 0.03; day 14, MV/CTB versus negative control: NS. (c) MV-specific endpoint titres of IgG1, IgG2a, IgG2b, IgG3 of individual mice immunized with 600 HAU MV (+CTB) and tested on day 49. Truncated titres are between 1:185216 and 1:200000. In (c) each group of four bars (groups separated by ticks) the first bar represents IgG1 titre, the second IgG2a titre, the third IgG2b titre and the fourth, IgG3 titre.

Flow cytometry. The MV-specific monoclonal antibodies (MAbs) were a gift of Dr F. Wild (Institut Pasteur, Lyon). The Ltk- cells transfected with the MV-H and MV-F protein were produced by one of us (Beauverger et al., 1993, 1994) in the laboratory of Dr Wild. The cells were grown to subconfluency in Dulbecco's modified Eagle's medium substituted with the additives used in the Vero cell cultures (see MV preparation). The cells were mechanically harvested using a rubber policeman, washed and aliquots of 5 x 10^6 cells were incubated on ice for 30 min with 100 μl of 1:40 diluted mouse serum or with saturating concentrations of MV-H (35) and MV-F (263-5) specific MAbs. Cells incubated with control serum, irrelevant antibody or no antibody, served as negative controls. After washing the cells with FML medium the cells were labelled with an FITC conjugate of goat anti-mouse IgG or IgA antibody. All cell washes or diluions of sera and MAbs were performed in FML medium (PBS containing 0.2 % sodium azide and 1% BSA). Five thousand cells were measured by flow cytometry on a Coulter EPICS instrument.

Statistical analysis. Statistical analysis was done using the ALMO Statistical Systems program (K. Holm, University of Linz, Austria). Differences between means of groups were analysed using a t-test for independent variables, by comparing log~ transformations of mean experimental titres. Statistical significance was considered when
The same program package was used for calculating the correlation coefficient, \( r \), and the level of significance, \( P \), of the correlation.

**Results**

*Serum anti-MV antibody subclasses*

The time course of the systemic antibody response was investigated after intranasal immunization of mice with 600 HA units MV in 10 \( \mu l \) PBS supplemented or not supplemented with 10 \( \mu g \) CTB using the standard intranasal protocol. Sera were obtained on different days and end-point titres of MV-specific IgG (Fig. 1a) and IgA (Fig. 1b) were determined.

Average IgG end-point titres of the MV/CTB groups were significantly higher (about 8 to 10-fold; \( P \) values in legend to Fig. 1) than in the corresponding MV group. After a single boost (day 28) with MV and CTB the IgG titres were higher (\( P < 0.02 \)) than after three boosts (day 97) with MV alone. Low levels of MV-specific IgA became detectable (\( > 1:25 \)) only after the third boost with MV, whereas titres of 1:200 and more were found after a single boost with MV/CTB (Fig. 1b). In the MV/CTB groups, systemic MV-specific IgA levels were about 100- to 200-fold lower than IgG levels. While the time course of IgG titres showed a steady increase after each boost with CTB, maximal IgA titres were found after the second boost with MV/CTB and tended to decrease, albeit not significantly (see results at day 97 and day 35) despite an additional boost on day 92. The late IgA increase after immunization with MV alone and the apparently transient response to MV/CTB explains why on day 97 the difference due to CTB is no longer significant (\( P > 0.4 \)). In the above experiments MV-specific titres of preimmune sera, and of IgA from animals immunized with PBS with or without CTB were invariably below the detection limit (data not shown).

Isotype analysis (Fig. 1c) showed that the response was 99% IgG1 in the MV groups. In the MV/CTB group up to 10% IgG2b and about 1% IgG2a were found in addition to the dominant IgG1. Specific IgG3 was below the detection limit in all animals.

*Haemagglutination-inhibiting (HI) and neutralizing (NT) antibodies*

To characterize functionally the immune response after intranasal immunization, the development of HI and NT activities were monitored. In the presence of CTB higher titres of HI antibodies developed after fewer boosts than after immunizing with MV alone (two boosts with MV/CTB and three boosts with MV: \( P < 0.02 \); three

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*Fig. 2.* Time profile of HI (a) and NT activities (b) after standard (intranasal) immunization with 600 HAU MV ± CTB. The bars represent reciprocal titres (see Methods) of individual mice. Lowest titres tested were 1:20 (HI) and 1:10 (NT). Sera tested negative were arbitrarily attributed a titre of 1:2 for graphical reasons. \( P \) values compare corresponding groups co-immunized with or without CTB.

(a) HI: day 14, NS; day 28, \( P < 0.02 \); day 35, \( P < 0.003 \); day 97, \( P < 0.003 \); day 35, \( P < 0.007 \); day 97, \( P < 0.0001 \). Dose-response curve (on day 35) of HI (c) and NT (d) titres after immunization on day 0, 14 and 28 with 0 to 2400 HAU MV in the presence or absence of 10 \( \mu g \) CTB. \( P \) values compare corresponding groups co-immunized with or without CTB and MV. (c) HI: 0 HAU MV, NS; 300 HAU MV, NS; 600 HAU MV, \( P < 0.003 \); 1200 HAU MV, \( P < 0.02 \); 2400 HAU MV, \( P < 0.005 \). (d) NT: 0 HAU MV, NS; 300 HAU MV, NS; 600 HAU MV, \( P < 0.03 \); 1200 HAU MV, \( P < 0.002 \); 2400 HAU MV, \( P < 0.02 \).
Measles virus mucosal immunization

Fig. 3. Comparison of endpoint titres of MV-specific IgG and IgA in the saliva (a), nasal wash (b), lung lavage (c) and gut wash (d) following the standard immunization protocol with 300–2400 HAU MV with or without 10 μg CTB (samples were taken on days 35, 97, 97 and 97, respectively). Data are expressed as log₂ transformations of reciprocal mean endpoint titres (by ELISA) of four mice. Lowest titres tested were 1:10 (nasal wash, lung wash and saliva) or 1:5 (gut wash) both for IgG and IgA. The following P values compare groups co-immunized with CTB with the corresponding groups immunized with MV alone. (a) Comparison with mock-immunized mice; saliva IgG: 600 HAU MV, P = ns; 1200 HAU MV, P = ns; 2400 HAU MV, P = <0.02; 600 HAU MV/CTB, P = <0.03; 1200 HAU MV/CTB, P = <0.05; 2400 HAU MV/CTB, P = 0.02; saliva IgA: 600 HAU MV, P = ns; 1200 HAU MV, P = <0.03; 2400 HAU MV, P = ns. Comparison of groups immunized with or without CTB, saliva IgG: 600 HAU MV, P = ns; 1200 HAU MV, P = <0.01. (b) Comparison to mock-immunized mice, nasal IgG: 300 HAU MV, P = ns; 600 HAU MV, P = ns; 1200 HAU MV, P = <0.05; nasal IgA: 300 HAU MV, P = ns; 300 HAU MV/CTB, P = <0.03; 600 HAU MV/CTB, P = <0.02; 1200 HAU MV/CTB, P = <0.004; comparison MV and MV/CTB (IgG): 300 HAU MV, P = <0.001; 600 HAU MV, P = <0.008; 1200 HAU MV, P = <0.02; lung IgG: 300 HAU MV, P = ns; 1200 HAU MV, P = <0.004. (c) Comparison to mock-immunized mice, lung IgG: 300 HAU MV, P = ns; 1200 HAU MV, P = <0.02; 300 HAU MV/CTB, P = <0.002; 600 HAU MV/CTB, P = <0.003; 1200 HAU MV/CTB, P = <0.002; 1200 HAU MV/CFA, P = <0.002; lung IgA: 300 HAU MV, P = ns; 600 HAU MV/CTB, P = <0.02; 1200 HAU MV/CTB, P = <0.02; 1200 HAU MV/CFA, P = <0.001; comparison MV and MV/CTB (IgG): 300 HAU MV, P = ns; 1200 HAU MV, P = <0.002; lung IgA: 300 HAU MV, P = <0.002; 600 HAU MV/CTB, P = <0.001; 1200 HAU MV/CFA, P = <0.002; comparison MV and MV/CTB (IgA): 300 HAU MV, P = <0.001; 1200 HAU MV, P < 0.05. (d) Comparison with mock-immunized mice, gut wash, IgG: MV/CFA P < 0.001; all other IgG and IgA levels are not significant. Immunizations received by the mice were: columns 1, 0 HAU MV; columns 2, 600 HAU MV; columns 3, 1200 HAU MV; columns 4, 2400 HAU MV; columns 5, 10 μg CTB; columns 6, 600 HAU MV+10 μg CTB; columns 7, 1200 HAU MV+10 μg CTB; columns 8, 2400 HAU MV+10 μg CTB; columns 9, 300 HAU MV+CFA; columns 10, 1200 HAU MV+CFA.

Boosts with MV/CTB and four boosts MV: P < 0.02. At an average HI titres were 5- to 10-fold higher (P values in legend to Fig. 2) in the MV/CTB group than in the corresponding group immunized with MV alone. The most significant effect of CTB was found when NT antibodies were analysed in the serum. Neutralization was found reliably only after co-immunization with MV and CTB. High titres were found after the third boost. Animals which were immunized with MV alone only occasionally developed low titres of neutralizing antibodies.

The dose-response of serum HI and NT antibodies was investigated after intranasal immunization with 0 to 2400 HAU of MV in the presence or absence of 10 μg CTB. Animals were boosted on day 14 and 28 and sera were obtained on day 35. In the presence of CTB the mean HI titres were 5- to 10-fold higher than in the absence of CTB (P values in legend to Fig. 2). In the presence of CTB the mean HI activity of 300 HAU was equivalent to immunizing with 1200 to 2400 HAU of MV alone. NT antibodies were found in all animals co-immunized with CTB and between 600 and 2400 HAU of MV. In the absence of CTB only low levels of NT antibodies were found in some animals immunized with higher doses of MV. When individual animals immunized with CTB and (different doses of) MV were compared a good correlation (r = 0.88; P < 0.0001) was found between their NT and HI activities.

Mucosal MV-specific antibodies

Mice were immunized and boosted three times according to the standard protocol using 1200 HAU MV with or without CTB (Fig. 3). Saliva was obtained on day 35 (i.e. after two boosts). Nasal, lung and gut washes were obtained on day 97 when the animals were killed. The strongest IgA and IgG responses were found in the saliva and nasal wash after co-immunization with MV and.
CTB. In these organs, CTB was required for significant levels of IgG and IgA: animals immunized with MV alone did not develop virus-specific antibody titres in the nasal wash and the saliva, which were statistically different from mock-immunized control mice (P values in legend to Fig. 3). IgG levels after nasal immunization with MV/CTB were of similar magnitude to those found after intraperitoneal (i.p.) immunization with 1200 HAU MV/CFA while MV-induced IgG levels were significantly lower (1200 HAU MV/CFA and 1200 HAU MV: P < 0.02). The intraperitoneal route did not induce significant levels of IgA (P > 0.05).

In the nasal wash, 600 or 1200 HAU MV induced significant levels of IgG and IgA only in combination of CTB. The IgG levels after intranasal MV immunization were lower than after i.p. immunization with MV/CFA (300 HAU MV, P < 0.0001; 1200 HAU MV, P < 0.001). The latter were comparable to MV/CTB induced titres (300 HAU MV, P < 0.02; 1200 HAU MV, P not significant). As in the saliva, consistent levels of IgA were found in the nasal wash after MV/CTB immunization but not after i.p. immunization. In contrast to the 100- to 200-fold differences between specific IgG and IgA in the serum, IgG and IgA titres in the saliva and the nasal wash were of similar magnitude.

In the lung wash there was no difference in IgG (i) between the groups immunized with or without CTB, (ii) after intraperitoneal immunization or nasal immunization with 1200 HAU MV/CTB. IgA is found after MV/CTB immunization but not after MV or MV/CFA immunization. In the experiment shown significance is only reached when the values from 600 HAU MV/CTB and 1200 HAU MV/CTB are combined, because of high standard deviations.

In the gut wash, specific IgG or IgA levels are not significantly elevated in comparison to levels in mock-immunized animals. Significant levels of IgG are found only after i.p. immunization (P < 0.001). The above secretions did not contain measurable levels of HI or NT activities.

**Specificity of antibodies**

To detect antibodies reacting with native MV-H and MV-F protein, transfected Ltk<sup>−</sup> cells were incubated with sera obtained on day 35 from mice immunized with 1200 HAU MV in the presence or absence of CTB. Antibody binding was measured by flow cytometry. Polyclonal antibodies obtained from mice immunized and boosted with MV emulsified in complete/incomplete Freund's adjuvant as well as monoclonal anti-MV-H and -F antibodies served as positive and/or negative controls. Fig. 4 shows that sera both from MV and MV/CTB immunized mice (standard protocol) react...
with the Ltk- cells transfected with H but not with F protein. The reactivities of the polyclonal and the relevant monoclonal antibodies demonstrate that H-transfected cells expressed more heterologous protein than the F-transfected cells. Despite the higher sensitivity of the assay for the detection of anti-H antibodies it can be concluded that little or no anti-F antibody seems to be generated after intranasal immunization. The reactivity of the sera from intraperitoneally immunized mice demonstrated the integrity of the MV preparation with respect to F protein.

**Gastric immunization**

In parallel experiments animals were immunized intragastrically with 10 μg cholera holotoxin B or heat-labile holotoxins of *E. coli* (LT) as adjuvants in combination with 50 μg MV using the time schedule of the standard protocol for immunization and bleeding. Four to six days after the 3rd boost, sera, nasal wash, saliva, lung washings and gut washings were harvested and tested for MV-specific antibodies by ELISA, for NT and HI antibodies and by Western blot for antibodies against denatured virus. No consistent MV-specific reactivity was detected after intragastric immunization when LT or CTB was used (data not shown).

**Antibodies to CTB**

All animals immunized intranasally with CTB, whether in the presence of virus or not, generated mean specific antibody titres to CTB of 1:0.5 × 10⁶ (range: 1:0.25 × 10⁶ to 1 × 10⁶). This confirms that all antigen preparations were effective even in those groups where no response to the MV was detected.

**Discussion**

Passively acquired maternal antibodies fully protect infants against natural measles virus infection (Black, 1989). In animal studies, neutralizing MAbs can prevent MV encephalitis (Giraudon & Wild, 1985). This underlines the importance of systemic antibodies in immune protection against measles, even though cell-mediated immune responses have a dominant role in clearing the MV infection (de Vries et al., 1988; Niewiesk et al., 1993) and are probably required for long-lasting protection. The present study was designed to investigate the antibody reaction to the MV after immunization by different mucosal routes and to test whether the response can be enhanced by the adjuvant effect of enterotoxins.

After a course of three intranasal immunizations, MV induced virus-specific IgG but no IgA and low levels of HI but no NT antibodies in the serum. When the MV was mixed with CTB, less antigen was required for a similar response and significantly higher IgG, IgA, HI and NT titres developed after fewer antigen boosts. In fact four- to sixfold less MV was required in the presence of CTB to induce similar HI titres. NT antibodies were only found in the MV/CTB groups. NT titres correlated with HI titres (r = 0.85) and with IgG (r = 0.78) but not with IgA titres (Table 1) suggesting that NT activities were mainly associated with IgG antibodies. In the serum, no IgA was detected after several immunizations with MV alone; after immunization with MV and CTB a maximal IgA response was observed around day 35 (7 days after the second boost). Despite an additional boost on day 92, serum IgA levels continued to decrease. This is compatible with the more transient nature of the IgA response at least on mucosal surfaces (Friedman et al., 1989) and the concept that some of the serum IgA may be of mucosal origin (Burlington et al., 1983).

Table 1. Correlation between serum IgG, IgA, HI and NT activities of animals immunized with MV/CTB (upper-right triangle) and of animals immunized with MV alone (lower-left triangle)

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<th>MV + CTB</th>
<th>MV alone</th>
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<td>IgG 0.73 (0.001*)</td>
<td>0.78 (0.01)</td>
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<td>IgA 0.89 (0.001)</td>
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<td>HI 0.80 (0.001)</td>
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<td>NT 0.85 (0.001)</td>
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*Correlation coefficient r (P value).
NS, Not significant.

Binding studies with the above sera and native F and H protein expressed on transfected Ltk- cells showed that the MV-specific IgG was directed against the MV-H protein. These findings are in line with the notion that NT antibodies are directed primarily against MV-H protein (Giraudon & Wild, 1985; Sato et al., 1985) and to a lesser extent against the MV-F protein (Malvoisin & Wild, 1990).

Thus, despite an adequate anti-F response after intraperitoneal immunization, the nasal route did not generate detectable antibodies against this immunodominant surface protein. The absence of a humoral response to MV-F was also observed in humans who were immunized subcutaneously with an inactivated MV vaccine. This suggests that the inability of the MV to replicate in the host rather than the lack of antigenic F protein seems to prevent the formation of anti-F antibodies. In contrast to our results with the MV, nasal immunization with killed respiratory syncytial virus (RSV) generated a 7- to 10-fold higher IgG response against glycoprotein F than against the other surface protein, G (Reumann et al., 1991a, b). In humans, the
insufficient antibody response to the F protein of both MV (Norby et al., 1975; Merz et al., 1980) and RSV (Kapikian et al., 1969) is thought to be the cause of atypical disease after invasive immunization with inactivated virus and exposure to virulent virus. The understanding of the immunopathology of atypical measles is important for the assessment of new measles vaccines. Therefore, the question of whether the latter phenomenon shares pathophysiological aspects with the incomplete response to nasally administered MV deserves further attention.

Isotype analysis showed that the specific antibodies were primarily of the T cell-dependent IgG1 type which is characteristic for the immune response to soluble proteins (Rosenberg & Chiller, 1979); specific IgG3, which is usually restricted to carbohydrate antigens (Perlmutter et al., 1978), against the MV surface glycoproteins was not detected. IgG2a is typically generated in virus-infected mice i.e. in response to proliferating virus such as RSV (Reuman et al., 1991 a, b) or Sendai virus (Coutelier et al., 1988). Only 1 to 2% of specific IgG2a was found in the 600 HAU MV/CTB group only. This corresponds to the fact that the mouse is not a natural host of MV and that proliferation is at best very limited (Niewiesk et al., 1993). However, our findings are in contrast to observations with the mouse-permissive RSV which induces primarily an IgG2a response even after virus inactivation (Reuman et al., 1991 b). The main isotype difference between the MV and MV/CTB group was the presence of up to 10% of specific IgG2b in the latter animals despite much higher titres of specific total IgG and none in the former. In the absence of CTB no specific IgG2a/b was produced.

Comparisons of titres between nasal washes, lung lavages and sera are biased because of the dilution with washing fluids (estimated at 1:100 in the nasal washes and 1:20 to 1:40 in the lung washes). In the saliva, nasal and lung washes IgG levels after immunization with 600 or 1200 HAU MV + CTB were comparable to those after i.p. immunization with CFA. CTB was required to induce virus-specific IgA in the nasal wash and the saliva, but no significant effect of the adjuvant was found in the lung wash or the gut wash. After i.p. immunization no IgA was detected on the mucosal surfaces or in the serum.

CTB has been used as a mucosal adjuvant with a number of proteins but the experience with co-immunization with whole virus is limited. A comparison of different studies is complicated by differences in schedule, doses and antigen preparations. Killed RSV induced IgA and IgG in the lung and nasal secretions as well as IgG in the serum after intranasal immunization of BALB/c mice (IgA in the serum was not tested for; Reuman et al., 1991 a, b). Similar to our results with the MV and CTB, co-immunization with CT induced a stronger and faster local and systemic response (Reuman et al., 1991 a). It is interesting to note that in the latter study, CT mediated an immune response to inactivated virus which was similar to that after live RSV infection. After intranasal immunization of BALB/c mice with whole killed influenza A, virus-specific antibodies were found in the serum, saliva (IgA; Novak et al., 1993) and in the broncholar lavage (IgG and IgA; Cao et al., 1992). To obtain similar results with the MV, CTB as an adjuvant was required. However, further studies with recombinant CTB will be required to exclude the possibility that contamination of subunit A may be partially responsible for some of the effects.

If CTB was effective after nasal administration no specific antibodies were detected after intragastric immunization whether CTB was used or not. As with MV, the nasal route was more effective than the oral immunization when CTB was used as adjuvant to immunize BALB/c mice against influenza (Hirabayashi et al., 1990) or Sendai virus (Liang et al., 1988). Our findings are in line with these and other observations that BALB/c mice are low responders after intragastric immunization. However, when in the latter study CTB was substituted by the holotoxin local and systemic antibodies were induced (Liang et al., 1988; Nedrud et al., 1987). Successful oral immunization has also been achieved with sonicated Helicobacter pylori and the cholera holotoxin in outbred Swiss mice (Czinn et al., 1993) or with inactivated influenza virus and the heat-labile enterotoxin of E. coli in BALB/c mice (LT; Chen & Quinnan, 1988). Even in the presence of the LT holotoxin (Clements et al., 1988), intragastric MV was unable to elicit antibodies detectable by ELISA or Western blot.

This is one of the first studies of mucosal immunity to MV in an animal model. It adds further evidence to the observations made with RSV, influenza and Sendai virus that the mucosal immunity and the adjuvant effect of enterotoxins are difficult to predict even in related viruses. The disparate reaction of the mucosal immune system is not due to virus proliferation but rather to properties of the viral proteins. The results from mouse studies may not be fully applicable to immunizations of humans with live measles vaccines since mice are not natural hosts of the MV. However, these animals are useful for studying the immunogenicity of non-replicating MV antigens administered by different routes.

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