In order to study the mucosal and serum antibody response to polysaccharide-encapsulated bacteria in mice, a preparation of heat-inactivated *Streptococcus pneumoniae* type 4 was administered, with and without cholera toxin, at various mucosal sites. It appeared that intranasal immunization of nonanesthesized animals was superior to either oral, gastric, or colonic-rectal antigen delivery with regard to the induction of serum immunoglobulin G (IgG) and IgA, as well as saliva IgA antibodies specific for pneumococci. The marked IgA antibody response in feces after intranasal, but not after oral or gastric, immunization is suggestive of a cellular link between the nasal induction site and the distant mucosal effector sites. Intranasal immunization also induced antibodies in serum and in mucosal secretions against type-specific capsular polysaccharide. IgA and IgG antibody levels in pulmonary lavage fluids correlated well with saliva IgA and serum IgG antibodies, respectively. Antibody determinations in pulmonary secretions may therefore be redundant in some cases, and the number of experimental animals may be reduced accordingly. After intraperitoneal challenge with type 4 pneumococci, mice immunized intranasally were protected against both systemic infection and death, even without the use of cholera toxin as a mucosal adjuvant. Thus, an efficient intranasal vaccine against invasive pneumococcal disease may be based on a very simple formulation with whole killed pneumococci.
Nasal immunizations are most efficient for induction of systemic and mucosal antibodies. To find the most efficient means of applying mucosal vaccines consisting of whole killed pneumococci, groups of mice were immunized via the nasal, oral, gastric, or rectal route. It was evident from the results of this experiment that the nasal route, with CT as a mucosal adjuvant, was by far superior to any other route for induction of serum IgG antibodies to whole pneumococci (Table 1). No such antibody response was elicited when the pneumococcal

TABLE 1. Concentration of IgG antibodies after immunization with heat-inactivated pneumococci plus CT by various mucosal routes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc after immunization by indicated routea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>2.107** (1.507–3.874)</td>
</tr>
<tr>
<td>Saliva</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>Lung lavage fluid</td>
<td>5.1* (3.4–9.3)</td>
</tr>
<tr>
<td>Extracts of feces</td>
<td>3.3** (1.8–6.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oral</th>
<th>100 (100–383)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric</td>
<td>127 (100–315)</td>
</tr>
<tr>
<td>Rectal</td>
<td>226 (100–370)</td>
</tr>
<tr>
<td>Controls</td>
<td>100 (100–565)</td>
</tr>
</tbody>
</table>

a Median concentrations (ranges) of IgG antibodies to whole pneumococci (kiloiuunits per milliliter or kiloiuunits per gram of dry feces). **, P ≤ 0.005; *, P ≤ 0.05 (statistical significance versus the control group; Mann-Whitney U test).

RESULTS

Nasal immunizations are most efficient for induction of systemic and mucosal antibodies. To find the most efficient means of applying mucosal vaccines consisting of whole killed pneumococci, groups of mice were immunized via the nasal, oral, gastric, or rectal route. It was evident from the results of this experiment that the nasal route, with CT as a mucosal adjuvant, was by far superior to any other route for induction of serum IgG antibodies to whole pneumococci (Table 1). No such antibody response was elicited when the pneumococcal...
antigen was given via other routes, even when antigen was
given directly into the stomach with bicarbonate to neutralize
the gastric acid. However, all immunized mice belonging to
any group developed strong serum IgG antibodies to CT (results
not shown). In the second experiment, in which whole killed
pneumococci were given intranasally with or without CT, levels
of serum IgG and IgM antibodies to whole pneumococci were
markedly higher in mice which were given pneumococci with
CT than in those given pneumococci without CT (data not
shown). The advantage of nasal immunization, compared to immu-
nization via other mucosal routes, was likewise clearly evident
for induction of IgA antibodies to whole pneumococci in se-
rum, as well as in saliva, lung lavage fluid, and extracts of feces
(Table 2). Presentation of the antigen into the lower part of the
intestine via the rectal route, however, also induced consistent
increases in fecal IgA antibodies to pneumococci (Table 2),
and rectal, as well oral, immunizations led to systemic IgA
antibody responses.

IgA antibodies in saliva and IgG antibodies in serum cor-
relate with the corresponding antibodies in pulmonary lavage
fluid. Only low concentrations of IgA antibodies to pneumo-
cocci were found in lung lavage fluid. Still, a significant in-
crease in IgA antibodies was observed in the lung lavage fluid
in the groups of mice which had been immunized via the nasal
or oral route (Table 2). Moreover, the concentration of IgA
antibodies in lung lavage fluid correlated well with the con-
centration of IgA antibodies in saliva (Fig. 1, upper panel) ($r =
0.89; P < 0.0001$) but with concentrations roughly 10 times less
than those in saliva. On the other hand, IgA antibody concentra-
tions in lung lavage fluid did not correlate significantly with
serum IgA concentrations.

Lung lavage fluid, as well as extracts of feces, contained
relatively high concentrations of IgG antibodies, especially in
the group of mice which had been immunized via the intrana-
sal route (Table 1). IgG antibodies to pneumococci in lung
lavage fluid correlated well with the corresponding serum IgG
values (Fig. 1, lower panel) ($r = 0.93; P < 0.0001$), although the
absolute concentrations in the pulmonary secretions were at
least 100 times lower than those in the sera.

Nasal immunization with whole killed pneumococci can in-
duce antibodies to PPS. In the first immunization experiment,
with the use of CT as a mucosal adjuvant with BALB/c mice,
IgG antibodies to PPS could not be detected in serum or
secretions. However, significant increases in serum IgA anti-
bodies to PPS could be demonstrated after nasal, oral, gastric,
and rectal immunization (Table 3). On the other hand, extracts
of feces were the only kind of sample representing secretions in
which such IgA antibody responses could be demonstrated,
and then only after nasal and rectal antigen deliveries (Table
3). Significant increases in serum IgM antibodies to PPS were
detected after intranasal oral, gastric, and rectal immunization,
but no IgM antibodies to PPS could be demonstrated in any
secretion (results not given).

The second immunization experiment, in which whole killed
pneumococci were given intranasally, with or without CT as a
mucosal adjuvant, to NIH mice, confirmed that serum anti-
PPS antibodies could be elicited. In this experiment, animals
responded with both IgG and IgM antibodies to PPS when CT
was used ($P \leq 0.01$ and $P < 0.001$, respectively) (Fig. 2). Some
mice showed an increase in IgA antibody levels, but the in-
crease was not statistically significant. When pneumococci

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nasal$^a$</th>
<th>Oral$^b$</th>
<th>Gastric$^c$</th>
<th>Rectal$^d$</th>
<th>Controls$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>103.3***</td>
<td>31.3***</td>
<td>2.9 (1.7–6.4)</td>
<td>28.4**</td>
<td>1.5 (6.4–1.2)</td>
</tr>
<tr>
<td>Saliva</td>
<td>5.5***</td>
<td>2.3*</td>
<td>0.1 (0.1–0.2)</td>
<td>0.1 (0.1–0.3)</td>
<td>0.1 (0.1–0.1)</td>
</tr>
<tr>
<td>Lung lavage fluid</td>
<td>0.8*</td>
<td>0.7*</td>
<td>0.1 (0.1–0.1)</td>
<td>0.1 (0.1–0.2)</td>
<td>0.1 (0.1–0.1)</td>
</tr>
<tr>
<td>Extracts of feces</td>
<td>143.0***</td>
<td>33.0</td>
<td>10.0 (10.0–19.1)</td>
<td>310.8***</td>
<td>10.0 (10.0–28.0)</td>
</tr>
</tbody>
</table>

$^a$ Median concentrations (ranges) of IgA antibodies to whole pneumococci (kilo-units per milliliter or kilo-units per gram of dry feces); ***, $P \leq 0.005$; **, $0.005 < P \leq 0.01$; *, $0.01 < P \leq 0.05$ (statistical significance versus the control group; Mann-Whitney U test).

$^b_n = 6$.

$^c_n = 4$. 

**FIG. 1.** (Upper panel) Correlation between concentrations of IgA antibodies to whole pneumococci (WC) in lung lavage fluid and saliva from mice which had been immunized via different routes with heat-inactivated pneumococci type 4 plus CT ($r = 0.89; P < 0.0001$). (Lower panel) Correlation between concentra-
tions of IgA antibodies to whole pneumococci type 4 in serum and lung lavage
fluid from the same mice ($r = 0.93; P < 0.0001$).
alone were used for nasal immunizations, however, only IgM antibodies to PPS were increased ($P < 0.001$). Moreover, there was no significant difference in the IgM antibody levels whether or not CT was added to the antigen solution (Fig. 2).

**Nasal immunizations with whole killed pneumococci can protect against systemic pneumococcal infection.** The groups of mice which had been immunized intranasally as part of the second experiment, and which were given lethal doses of viable pneumococci intraperitoneally, were examined for viable bacteria in the blood. As early as 3 h after the bacterial challenge, the animals which had been immunized with the pneumococcal preparation had significantly ($P < 0.01$) fewer bacteria than those which had been given only saline as a nasal placebo vaccine (Fig. 3). It made no difference whether CT had been given together with the killed pneumococci. This difference in bacterial counts between control animals and animals which had been given pneumococci with or without CT was even more pronounced at 12 h after the bacterial challenge ($P < 0.001$).

None of the mice which had been given saline as a placebo vaccine survived the first 2 days after challenge with viable bacteria (Fig. 4). On the other hand, all except one mouse in each of the two groups which had been given the killed pneumococcal vaccine intranasally survived the whole observation period of 2 weeks. The protective effect of this nonproliferating nasal vaccine has thus been confirmed to include severe life-threatening pneumococcal sepsis. Moreover, pneumococci alone were sufficient to attain this effect, i.e., it did not seem necessary to include the commonly used CT as a mucosal adjuvant.

**DISCUSSION**

In this study, we have shown that whole heat-inactivated pneumococci can induce both systemic and mucosal antibodies when applied on various mucosal surfaces. Results of our first experiment indicate that intranasal application of this antigen, plus CT as a mucosal adjuvant, was superior to the oral, gastric, and rectal routes of antigen delivery (2). It was also evident

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**TABLE 3. Concentrations of IgA antibodies after immunization with heat inactivated pneumococci plus CT by various mucosal routes**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nasala</th>
<th>Oralb</th>
<th>Gastricb</th>
<th>Rectalb</th>
<th>Controlsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.34*** (0.29–0.80)</td>
<td>0.30*** (0.26–0.33)</td>
<td>0.34*** (0.29–0.47)</td>
<td>0.46** (0.38–0.55)</td>
<td>0.12 (0.07–0.22)</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.26 (0.11–1.12)</td>
<td>0.17 (0.15–0.18)</td>
<td>0.17 (0.15–0.21)</td>
<td>0.18 (0.15–0.31)</td>
<td>0.15 (0.11–0.39)</td>
</tr>
<tr>
<td>Lung lavage fluid</td>
<td>0.32 (0.24–0.33)</td>
<td>0.28 (0.22–0.59)</td>
<td>0.30 (0.27–0.32)</td>
<td>0.29 (0.23–0.37)</td>
<td>0.34 (0.32–0.38)</td>
</tr>
<tr>
<td>Extracts of feces</td>
<td>0.56*** (0.25–1.65)</td>
<td>0.11 (0.07–0.33)</td>
<td>0.22 (0.08–0.36)</td>
<td>0.28** (0.15–0.99)</td>
<td>0.14 (0.11–0.19)</td>
</tr>
</tbody>
</table>

*Median concentrations (ranges) of IgA antibodies to pneumococcal polysaccharide type 4 (absorbance); ***, $P \leq 0.005$; **, 0.005 $< P \leq 0.01$ (statistical significance versus the control group; Mann-Whitney $U$ test).

$b n = 6.$

$c n = 4.$

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**FIG. 2. Serum antibodies to PPS 4 in mice immunized intranasally with heat-inactivated pneumococci type 4, with CT (+CT; eight mice), or without CT (–CT; six mice). Immunizations (v) were carried out at weekly intervals (days 0, 7, 14, and 21), and blood samples were taken on days 0, 14, and 28. A control group (seven mice) was immunized intranasally with saline (NaCl). The symbols represent individual levels (absorbance) of IgG, IgM, and IgA antibodies.

**FIG. 3. Bacteremia, expressed as number of CFU per milliliter of blood from individual mice after intraperitoneal challenge with 10 times the LD_{50} of live virulent pneumococci type 4 (~20 CFU/mouse). Groups of mice were immunized intranasally either with whole heat-inactivated pneumococci type 4 mixed with CT (solid diamonds) or with the same antigen without CT (solid circles) or were not immunized (open circles). The dotted line indicates the limit of detection of bacteremia. The mice were challenged 1 week after the fourth nasal immunization dose, which corresponds to day 28 in Fig. 2.
that immunization via these alternative routes was not able to induce anti-pneumococcal IgG antibodies, which might be crucial for protection against systemic disease. However, IgG antibodies against CT were induced after immunization at all mucosal sites. The induction of systemic immunity to pneumococci via the nasal route suggests that the nasopharyngeal mucosa possesses the necessary structures to make mucosal immunizations a realistic alternative to the use of parenteral vaccines (11).

The superiority of nasal versus oral, gastric, and rectal routes of antigen presentation was confirmed by the demonstration of specific IgA antibodies in serum and samples representing secretions. The demonstration of IgA antibodies in secretions after the antigen was given orally, and not gastrically, might thus be explained by the antigen in the first case having reached the nasopharyngeal induction sites. Furthermore, only intranasal immunizations, in addition to rectal antigen delivery, induced significant increases in intestinal IgA antibodies, as reflected in extracts of feces. This was surprising, considering the fact that neither oral nor gastric immunizations with the same antigen were able to induce significant increases in such intestinal antibodies. The lack of intestinal antibodies after oral and gastric immunizations indicates that induction of intestinal antibodies after intranasal immunization was not due to swallowing or leakage of antigen from the nose into the intestines. The stimulus for antibodies to be produced locally in the gut is therefore suggestive of a cellular link between the nasal induction site and the intestinal effector site.

Our finding of IgG as well as IgA antibodies to pneumococci in lung lavage fluid, especially after nasal immunization, might indicate that both these antibodies have a barrier function against invasive pneumococci. The IgG antibodies in the pulmonary lavage fluids also seemed to mirror antibodies in serum, from which they are probably derived. Similarly, we have recently shown that IgG antibodies to B. pertussis in pulmonary secretions reflected the corresponding serum antibodies, which were initiated by nasal immunization (9). A protective effect of systemic antibodies against pulmonary infections might thus be conferred all the way from the tissue fluid to the mucosal surfaces.

The present finding that pulmonary IgA antibodies to pneumococci correlated with such antibodies in saliva indicates that at least some of the IgA is produced locally in the lungs to contribute to this presumed surface protection. It seems, therefore, that the IgA antibodies in saliva reflect the IgA antibodies in the lung secretions and that analyses of salivary IgA would be sufficient for evaluation of mucosal airway antibodies. Since saliva samples can be collected several times from the same animal, there is less need for collection of pulmonary secretions, and the number of mice used for experimental purposes can be reduced.

In the second experiment, significant increases of serum anti-polysaccharide IgG and IgM were induced in NIH III mice, whereas only serum IgM antibodies were induced in the first experiment with BALB/c mice. Parenteral immunization of BALB/c mice with PPS, conjugate vaccine, or heat-inactivated pneumococci also seems to induce serum IgM and no IgG antibodies (3, 5, 19). In other strains of mice, however, IgG antibodies can be induced after parenteral immunization with a pneumococcal conjugate vaccine (19), and low levels of IgG antibodies may even be induced in NIH III mice after immunization with polysaccharides alone (unpublished observations). The discrepancy in antibody responses in the first versus the second experiment may therefore be due to the use of different strains of mice.

Intranasal immunization with PPS type 3 containing liposomes has also been shown by others to induce IgA antibodies specific for type 3 polysaccharide in lung lavage fluid (6). In addition, we have now shown that both nasal and rectal immunizations induced intestinal IgA antibodies directed against the homologous type of polysaccharide. To some extent, however, these intestinal antibody responses to the polysaccharide antigens after mucosal immunization seemed to depend on the use of strong mucosal adjuvants, such as CT, and it remains to be shown whether polysaccharides as part of a mucosal vaccine induce protective immunity.

The intranasally immunized mice in the present study were well protected against lethal intraperitoneal challenge with live pneumococci. This was evident also with a vaccine consisting of only killed whole pneumococci, i.e., without additional mucosal adjuvants, despite the fact that some mice developed only low levels of antibody to polysaccharides in the serum. A similar discrepancy between low levels of ELISA antibodies specific for serotype 4 polysaccharide and protection against pneumococci of the same serotype has also been observed in other studies (4, 19). As opposed to antibodies against polysaccharides, in the present study, intranasal immunization induced strong antibody responses to whole pneumococci in all mice, even without CT. Antibodies to pneumococcal antigens other than polysaccharide antigens may therefore have contributed to protection. Since such antigens may be common to many pneumococcal serotypes, one could speculate whether a pneumococcal whole-cell vaccine given intranasally would protect against infections caused by several other serotypes.

The present finding that CT was not necessary for a nasal whole-cell vaccine to induce effective antibodies is in accordance with results of studies with outer membrane vesicles from meningococci (14). Recently, it has been found that CT actually inhibited the antibody responses to whole group B streptococci (18) and to B. pertussis (9) that had been given intranasally. It may thus be possible to create effective nonproliferating mucosal vaccines based on very simple formulations.

ACKNOWLEDGMENTS

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REFERENCES


