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Anticarrier Immunity Suppresses the Antibody Response to Polysaccharide Antigens after Intranasal Immunization with the Polysaccharide-Protein Conjugate

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We have conjugated cholera toxin (CT) B subunit (CTB) to dextran and studied the effect in mice of previous immunization with CT and CTB on the response to dextran after intranasal immunizations with conjugate. Preexisting immunity to CTB was found to inhibit both the lung mucosal response and serum antibody response to dextran, but this effect could be overcome by using a higher dose of conjugate and delaying the conjugate immunization until the CTB antibody titers had declined. The role of anti-CTB antibodies on the mucosal surface was probably to prevent uptake of the conjugate through a mechanism of immune exclusion. Passively transferred serum antibodies against CTB, on the other hand, suppressed both the serum response and the local antibody response against CTB but did not affect the response to dextran after intranasal immunization with conjugate.

Mucosal immunity is important in protection against several pathogens (9, 11), and topical vaccination is often required for induction of effective local immunity (8, 26, 34). Today, with only few exceptions, vaccines licensed for human use are intended for parenteral injections and consequently induce mainly a systemic immune response. Most nonreplicating antigens are poor mucosal immunogens. It has been shown that mucosal immunogenicity is greatly facilitated by the ability of the antigens to bind to the mucosal surface either by themselves or via coupling to a binding agent (2, 8, 12, 20, 25, 26). Cholera toxin (CT) and its B subunit (CTB) are examples of exceptionally potent mucosa-binding immunogens and have been shown to induce strong immune responses after oral, nasal, or vaginal immunizations (8, 16, 21–23, 32). CT and CTB bind to the GM1 ganglioside receptor which is present on most nucleated mammalian cells (16), and both CT and CTB have been used successfully as carriers for various other antigens in order to improve their mucosal immunogenicity (8, 12, 20, 26, 32).

Polysaccharides are T-cell-independent antigens which are generally rather poor immunogens, but their immunogenicity can be significantly augmented by conjugation to a carrier protein (5, 28). Several pathogenic bacteria that enter the host via the respiratory route have a polysaccharide capsule, and it has been shown that anticapsule serum antibodies often can protect effectively against both infection and disease (1, 3). It is likely that antibodies against capsular antigens, if available at the mucosal surface, could help to prevent colonization and thus be able to protect against subsequent disease (6, 17, 33). Therefore, polysaccharide antigens are relevant vaccine candidates also for use in mucosal vaccines.

We have previously shown that it is possible to evoke a local antibody response in mice after intranasal immunization against a polysaccharide by conjugating it to CTB (8). The aim of this study was to investigate the influence of preexisting immunity, induced either by active intranasal immunization with the carrier or by passive administration of serum antibodies to the carrier on the response to the polysaccharide antigen in such conjugates. The first situation is of relevance for vaccine development using CTB as a carrier for different antigens, while the latter situation was chosen as an approximation of the effect of maternally acquired antibodies in infants.

The effect of preexisting immunity against the carrier protein on the antibody response to polysaccharide antigens after vaccination with polysaccharide-protein conjugates has been the subject of several studies in relation to parenteral vaccination. It has been reported that preimmunization with the carrier protein (tetanus or diphtheria toxoid) can either suppress (7) or enhance (15) immune responses against *Haemophilus influenzae* type b capsular polysaccharide in adult volunteers. However, this issue has not been studied in a mucosal context, e.g., after intranasal or peroral immunization.

MATERIALS AND METHODS

Chemicals. Recombinant CTB was purified from the extracellular medium after fermentor culture of *Vibrio cholerae* 358 containing the CTB-overexpressing plasmid pML-LCTB tac1, using a combination of hexametaphosphate precipitation and gel filtration as described previously (19). Dextran with an average molecular mass of 76 kDa was purchased from Sigma Chemical Co., St. Louis, Mo. The other reagents were *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pharmacia Fine Chemicals, Uppsala, Sweden); adipic acid dihydrazide (Fluka Chimie AG, Buchs, Germany); trinitro benzene sulfonic acid (Eastman Chemical Products Inc.); CT (List, Campbell, Calif.), heparin (Lövens kemiske fabrik, Ballerup, Denmark); dithiothreitol (Calbiochem Corporation, La Jolla, Calif.); and anthrone, avidin, CNBr, *N*-hydroxysuccinimidobiotin, and *o*-phenylenediamine (Sigma).

Conjugation. The dextran-CTB conjugate was prepared as described previously (8). Briefly, dextran was first derivatized with adipic acid dihydrazide by using CNBr activation and then conjugated to CTB by using the coupling reagent SPDP. The resulting conjugate was gel filtered on a Sephacryl S300 column (Pharmacia), and the peak corresponding to the highest-molecular-weight material was collected, concentrated, and stored with 0.005% merthiolate (Kebo AB, Stockholm, Sweden) at 4°C. The conjugate composition was 1.2 mg of dextran/ml and 0.64 mg of CTB/ml as determined by the anthrone reaction (29) and the Lowry protein determination method (Sigma). The GM1-binding capacity of CTB was not affected by the conjugation as determined by a GM1 enzyme-linked immunosorbent assay (ELISA) (8, 30).

Immunization and sampling. Intranasal immunizations of groups of five to seven 6- to 8-week-old female C57BL mice (B&K Universal, Solna, Sweden) were done as described previously (8). Before each immunization, a blood sample was taken from the tail vein. One microgram of CT was used as an adjuvant for all intranasal immunizations since it has been shown to improve the antibody response against CTB-conjugated antigens as well as unconjugated protein antigens, although the adjuvant effect is much smaller than after immu-

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TABLE 1. Immunization schedule

Group	Priming[a]	Booster (μg of conjugate/dose)	
		After 2 wk	After 3 mo
1	CTB	3	
2	CTB		3
3	PBS[b]	3	
4	CTB	15	
5	CTB		15
6	PBS	15	
7	CTB	75	
8	CTB		75
9	PBS	75	

[a] The CTB priming (25 μg/dose) was given on day 0 and 14, and the boosters were given 2 weeks or 3 months after the last priming.
[b] Sham immunized with PBS.

nizations by the oral route (21, 24). We have also shown that addition of CT gives a modest, (ca. twofold) increase in the antibody response against dextran after intranasal immunization with CTB-dextran conjugates (unpublished data). All conjugate immunization doses given refer to the polysaccharide. The experiments where mice were preimmunized intranasally with CTB and CT and then given different doses are summarized in Table 1. All of these groups consisted of six mice. For the experiments with passive administration of antibodies, groups of four mice were given 200 μl of antiserum intravenously in the tail vein 1 day before each of two intranasal immunizations with 15 μg of dextran conjugate. Consistent with the other experiments, the immunizations were given 2 weeks apart. One group received a pool of anti-CTB serum, and one group received a pool of antidextran-CTB serum. The antidextran immunoglobulin G (IgG) $\log_{10}$ titer was 3.6 in the antidextran-CTB serum, and the anti-CTB IgG $\log_{10}$ titer was 5.2 in both serum pools. The serum pools were obtained by immunizing mice systemically and on mucosal surfaces with either CTB or CTB-dextran conjugates. The mice were sacrificed 1 week after the last immunization unless otherwise stated. The antibody levels were determined in serum and in saponin extracts of lung tissue from extensively perfused sacrificed mice (8).

ELISA. For analysis of anti-CTB antibodies, GM1-coated plates were used (30); for antidextran antibodies, plates were coated with avidin and biotinylated dextran (8). All samples were titrated in threefold dilutions. The initial dilutions for serum samples were 1/10 and 1/100 and those for organ extracts were 1/3 and 1/30 for dextran ELISA and GM1 ELISA, respectively. In both ELISA methods, anti-mouse IgG or IgA horseradish peroxidase-conjugated antibodies (Jackson Immunoresearch Laboratories, West Grove, Pa.) were added and developed with *o*-phenylenediamine and H_2O_2. Results are given as the reciprocal dilution corresponding to an absorbance of 0.4 above the background. This cutoff was chosen as it is in the linear part of the titration curve.

Statistics. Statistical analyses were performed by using Statistica 4.0 for Windows (Softstat, Tulsa, Okla.). One-way analysis of variance (ANOVA) for repeated measures was used for evaluation of the dose-response study. Two-way ANOVA was used to evaluate the difference between the different immunization schedules. Post hoc comparisons of the individual groups were performed by Scheffé's test. The effect of passively transferred antibodies was evaluated by a one-way ANOVA. Values are given as mean ± standard error of the mean (SEM).

RESULTS

Dose-response study. A dose-response experiment was conducted to give guidance for the following experiments regarding the appropriate number of immunizations and the immunogenic dose. Groups of mice with five animals in each were given either 1, 10, or 50 μg of dextran in the form of conjugate in each of three nasal immunizations at 10-day intervals. Serum samples were collected immediately before each immunization and 1 week after the third dose, when the mice were killed and lung tissue extracts were also prepared. Analysis of antibody levels by ELISA showed that the serum IgG antidextran response to the 1-μg dose was significantly lower than the response to the 10- or 50-μg dose on day 17 ($P < 0.0001$). After three doses (day 27), the response to the 1-μg dose was significantly lower than the response to the 50-μg dose ($P = 0.0006$) but did not differ significantly from the response to the 10-μg dose (Fig. 1A). At the two higher doses of antigen, the antibody response reached a plateau value after two immunizations (Fig. 1A). The IgG and IgA antibody responses in the lungs were also dose dependent ($P = 0.01$ and $P = 0.04$) (Fig. 1B).

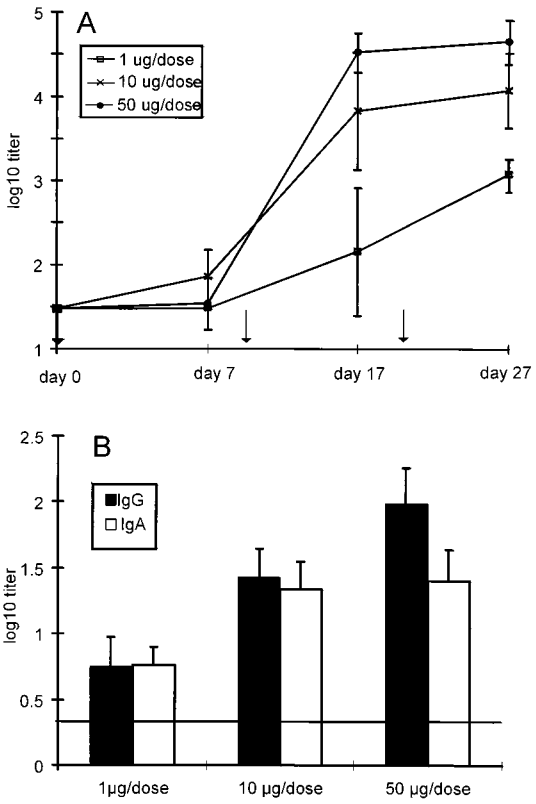

FIG. 1. (A) Serum IgG against dextran following three immunizations 10 days apart with different doses of dextran conjugated to CTB. The arrows indicate the days of immunization. (B) Lung IgG and IgA antidextran response following the last of three immunizations with different doses of dextran-CTB conjugate (B). Error bars represent SEM.

Effect of actively induced preexisting anticarrier immunity. Based on the dose-response results described above, we then tested the effect of preimmunization with a mixture of CTB and CT on the antibody response to the dextran component of different dosages of intranasally administered dextran-CTB conjugate given to groups of mice with six animals in each group. The mice were immunized twice intranasally with CTB-CT and, either 2 weeks or 3 months later, immunized twice intranasally with a low, intermediate, or high dose of dextran-CTB conjugate (Table 1). All mice had acquired high serum IgG and IgA anti-CTB titers ($\log_{10}$ titers of 6.05 ± 0.05 for IgG and 3.67 ± 0.18 for IgA) at 2 weeks after the two initial intranasal immunizations with CTB-CT. Three months after the CTB-CT immunizations, the serum anti-CTB titers had decreased to $\log_{10}$ titers of 5.44 ± 0.06 for IgG and 2.96 ± 0.15 for IgA. The IgG and IgA titers in the lungs were also high 2 weeks after the initial immunizations with CTB-CT ($\log_{10}$ titers of 4.92 ± 0.17 and 4.08 ± 0.25, respectively) and remained relatively high after 3 months (the $\log_{10}$ titers of 4.58 ± 0.11 and 3.66 ± 0.11, respectively). Control mice that were sham immunized with phosphate-buffered saline (PBS) did not have any detectable anti-CTB antibodies of either isotype in serum or the lungs. Since the local antibody titers could be deter-

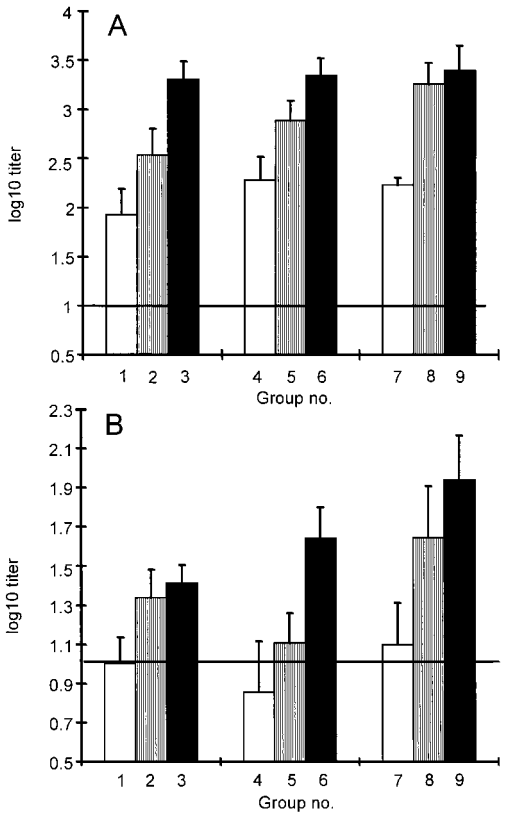

FIG. 2. Effects of preimmunization with carrier on the serum IgG (A) and IgA (B) antidextran responses to immunization with dextran-CTB conjugate. The groups are described in Table 1. White bars show groups preimmunized with CTB and immunized with conjugate 2 weeks later, gray bars show groups preimmunized with CTB and then immunized with conjugate 3 months later, and black bars show groups sham preimmunized with PBS and then immunized with conjugate. Error bars represent SEM. The preimmune $\log_{10}$ titers were <1.

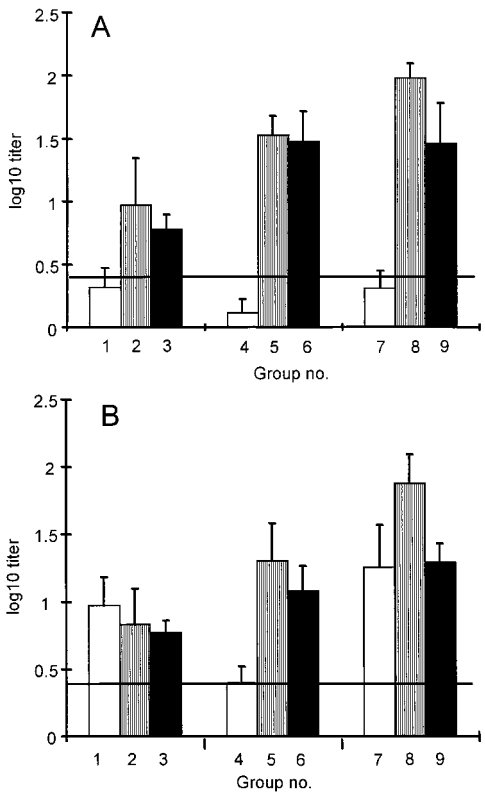

FIG. 3. IgG (A) and IgA (B) antidextran responses in extracts of the lungs. The groups are described in Table 1. White bars show groups preimmunized with CTB and immunized with conjugate 2 weeks later, gray bars show groups preimmunized with CTB and then immunized with conjugate 3 months later, and black bars show groups sham preimmunized with PBS and then immunized with conjugate. Error bars represent SEM.

mined only after the animals were sacrificed, these results were obtained from a separate group of mice.

As shown in Fig. 2A, all animal groups that were immunized with conjugate shortly after the CTB-CT immunizations (groups 1, 4, and 7) had significantly lower serum IgG antidextran levels than the concomitantly immunized animals which had not received any prior immunization (groups 3, 6, and 9) ($P = 0.0009$). The serum IgA titers were also significantly lower in the preimmunized groups ($P = 0.0004$) (Fig. 2B). After 3 months, however, the suppression of the serum IgG and IgA responses could be overcome partly or fully, especially by using a higher dose of intranasally administered conjugate ($P = 0.99$ for IgG and $P = 0.16$ for IgA compared with controls). Thus, preexisting anti-CTB immunity suppressed the serum antibody response to dextran when the conjugate was given intranasally 2 weeks after the CTB immunizations, but when the conjugate was given after 3 months, the IgG and IgA responses did not differ significantly from those in the control groups.

The antidextran responses in the lungs of the same groups of mice are shown in Fig. 3. The IgG response was significantly lower in the mice immunized with the conjugate 2 weeks after the CTB-CT immunizations than in the control groups ($P < 0.0001$). However, the IgA response did not differ significantly in the group immunized with the conjugate 2 weeks after the CTB-CT immunizations compared to the control group ($P = 0.63$). The mice preimmunized with CTB-CT 3 months before receiving conjugate responded as well as or slightly better than the control group with regard to both IgG and IgA antibodies ($P = 0.35$ and $P = 0.25$).

Effect of passively transferred serum antibodies. The effect of passively acquired serum antibodies was studied by giving mice anti-CTB or anti-CTB-dextran antiserum intravenously 1 day before each of two intranasal immunizations with dextran-CTB conjugate. These groups were compared with a concomitantly immunized control group which was not given any antiserum before immunization. As shown in Fig. 4A, the groups that received anti-CTB or anti-CTB-dextran antiserum developed significantly lower anti-CTB IgG as well as IgA responses in serum than the control group ($P < 0.0001$ for IgG and $P = 0.003$ for IgA), whereas the anti-dextran antibody response was not significantly different from that of the control group. The antibody responses in the lungs against CTB were also significantly suppressed ($P = 0.002$ for IgG and $P = 0.005$ for IgA), while the dextran responses were essentially unaffected (Fig. 4B).

DISCUSSION

Several capsular polysaccharide conjugate vaccines against *H. influenzae* type b have been licensed, and many other conjugate vaccines based on polysaccharides from, e.g., *Salmonella typhi* (31), *Streptococcus pneumoniae* (13), group B streptococci (18), and *Staphylococcus aureus* (14) have recently been developed and tested in animals and/or humans. There is, however,

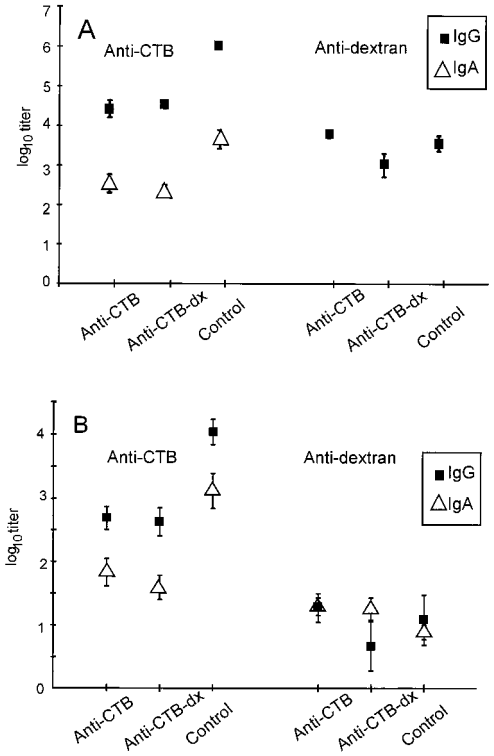

FIG. 4. IgG and IgA in serum (A) and in saponin extracts of lungs (B) against CTB and dextran in mice given antiserum intravenously before intranasal immunization. IgA in serum against dextran was not tested. The *x* axis indicates the specificity of the antiserum given to the mice before immunization. Error bars represent SEM.

limited information on the mucosal immunogenicity of such conjugates even though mucosal immunization with polysaccharide-protein conjugates able to attach to and be taken up by the mucosa should have great potential usefulness. Bacterial colonization of a mucosal surface is a prerequisite for many infections also with encapsulated bacteria, and prevention of colonization and subsequent invasion not only should be important for protection of the individual but also could decrease the risk for further spread of the infection within the population. Since antibodies against a bacterial polysaccharide capsule can prevent colonization (6, 33), we have found it important to study the mucosal immune response to appropriately formulated and administered polysaccharide-protein conjugates and, directly linked to this objective, identify suitable carrier proteins and immunization regimens for the purpose. Our work has been focused on the development of conjugate vaccines based on polysaccharide antigens coupled to CTB, and we have found that such conjugates appear to be better able than other formulations tested to generate mucosal and systemic immune responses to the coupled polysaccharide antigens (8). In the present study, we describe that preexisting immunity to the carrier protein, CTB, can substantially influence the outcome of the mucosal immunization with conjugate, and we have addressed how to overcome this effect.

The antibody responses to the model polysaccharide antigen-dextran in serum and lungs following intranasal immunization with dextran-CTB conjugate were suppressed when the mice were preimmunized with the carrier protein. This effect could be overcome by using a higher dose of conjugate and longer intervals between immunizations. One likely mechanism for this suppression is immune exclusion. While this is normally an important defense mechanism of mucosal antibodies against pathogenic microorganisms, preventing bacteria, viruses, and toxins from adhering to the mucosal surface (10), a similar immune exclusion mechanism could also interfere negatively with mucosal immunization efforts by blocking the administered antigen from uptake by the mucosal surface and thus leading to an impaired immune response. The anti-CTB antibody titers decreased over time, but after 3 months, substantial titers remained, both locally (46%) and in serum (38%). Similar antibody levels if present on the nasal mucosa itself could be sufficient for decreasing antigen uptake unless an excess of antigen was given. In contrast to the systemic antibody responses, there was a tendency toward higher antidextran responses in the lungs 3 months after priming with CTB than in controls. This may be explained by induction of local memory cells specific for CTB epitopes, thus providing extra help and enhancing the response to subsequent immunizations with conjugate. These results emphasize that the systemic and mucosal immune systems must be considered separately.

When anti-CTB antibodies were injected intravenously, however, the antibody response against dextran was not suppressed in serum or the lungs following intranasal immunization, whereas the antibody response against CTB was suppressed. Transfusion with an antiserum containing both anti-CTB and antidextran antibodies had a similar effect on the antibody responses as giving anti-CTB only. The antidextran titers were, however, lower than the CTB titers, and possibly use of a serum with a higher antidextran titer would give a different result. Taken together, the results indicate that the conjugates were taken up and reached antigen-presenting cells in spite of preexisting circulating anti-CTB antibodies and that the suppression in this instance was due to some other mechanism. There are several mechanisms by which passively acquired antibodies could suppress immune responses even after uptake via a mucosal surface, e.g., by blocking the antigen (CTB), opsonizing the conjugates for phagocytosis, and down regulating B cells by binding to their Fc receptors. The production of serum IgG against dextran in amounts that were not significantly different from those in the control group indicates that the dextran-specific B cells received significant T-cell help, probably from CTB-specific T cells. Whether these were induced to a lesser but still sufficient extent in mice with passively acquired antibodies cannot be ascertained from these data. It has been shown that in humans, anticarrier antibodies in serum can suppress the immune response against polysaccharides following systemic vaccination with conjugates (7). Sarvas et al. have also shown that IgG of maternal origin can suppress the immune response to vaccination in infants (27).

The effect of anticarrier immunity in a mucosal context has not been extensively studied, but in a study using the live vector *S. typhi* Ty21a expressing *V. cholerae* O antigen for oral immunizations, an inverse correlation between prevaccination *V. cholerae* antibody levels and titer increase was found. The antibody levels against the vector, on the other hand, did not influence the immune response (4). There are, however, fundamental differences in the mechanisms of uptake between live, particulate antigen and soluble conjugates, as well as differences in the mechanisms for immune induction in the gut and the upper respiratory tract. In summary, the results from this study are of importance for development of mucosal vaccines, and they emphasize the need to use dosages and regimens capable of overcoming reduced immunogenicity due to preexisting immunity to the carrier protein. An alternative approach to circumvent the problem could be to use different

carrier proteins in different vaccines, but today the number of suitable carrier protein candidates is limited.

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