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THE CELLULAR LOCATION OF A FOREIGN B CELL EPITOPE EXPRESSED BY RECOMBINANT BACTERIA DETERMINES ITS T CELL-INDEPENDENT OR T CELL-DEPENDENT CHARACTERISTICS[1]

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We have targeted two foreign B cell antigenic determinants to different locations in the *Escherichia coli* cell to examine what effect this had on antibody responses elicited by the recombinant bacteria. The two epitopes were the 132–145 peptide from the PreS2 region of hepatitis B virus and the C3 neutralization epitope of poliovirus type 1. They were each expressed in two forms either on the surface, as part of the outer-membrane protein LamB, or soluble in the periplasm, as part of the periplasmic protein MalE. When live bacteria expressing the foreign epitope at the cell surface were used for immunization of mice, they induced T cell-independent antibody responses characterized by a rapid induction of IgM and IgG antibodies. In contrast, when the same foreign epitope was inserted into the MalE protein, the antibody response was only detectable after 3 wk, belonged only to the IgG class and was strictly T cell dependent. This study has therefore identified two major pathways by which epitopes expressed by bacterial cells can stimulate specific antibody responses. The first pathway is mediated by direct activation of B cells by bacterial cell-surface Ag and does not require T cell help. The second pathway is T cell dependent and concerns Ag that can be released from the bacteria in a soluble form. We have also studied the effect of the exact position of the B cell antigenic determinant within the LamB protein and with respect to the outer membrane by comparing the immunogenicity of the PreS epitope inserted at three different permissive sites of LamB. The data indicated that to obtain an antibody response with intact bacteria, the epitope must be protruding sufficiently from the outside of the outer membrane. In contrast, when semipurified hybrid proteins were used as immunogen, the exact position of the B cell antigenic determinant within solubilized LamB protein does not influence its immunogenicity.

The mechanisms by which the immunogenicity of defined peptidic sequences is controlled are still poorly understood. So far, the relevant studies have been done almost entirely with soluble proteins. However, most of the Ag to which the immune system is exposed belong to complex and organized structures such as parasites or bacteria. In addition to the intrinsic characteristics of a given epitope, its exact location within these structures is likely to influence its immunogenicity. These environmental factors have been difficult to study so far due to the lack of appropriate experimental models.

By expressing the antigenic peptide of interest in "permissive sites" of bacterial proteins, it is possible to express the determinants without altering the cellular location or biologic function of the host proteins (1–4). This procedure allows the foreign peptide to be expressed within recipient proteins with different locations in the bacterial cell (intracellular targeting) or in different positions within a protein. As vector proteins we are using two *Escherichia coli* envelope proteins: LamB and MalE (1). Both are involved in the transport of maltose and maltodextrins into the cell. LamB is also the receptor for phage lambda and other phages. LamB is an integral outer membrane protein so that inserted foreign peptides are expressed in association with the outer membrane. In addition, because LamB spans the outer membrane a number of times and because permissive sites have been identified on both sides of the outer membrane, the foreign peptides can be exposed either at the cell surface or toward the periplasm (Fig. 1) (4). MalE is a periplasmic protein and inserted foreign peptides are expressed in a soluble form within the periplasm (5).

In previous work, we showed that antibody responses could be elicited against epitopes inserted in LamB sites exposed at the cell surface or within the MalE protein. We determined the influence of a number of critical factors such as the form of the immunogen (intact or killed bacteria or extracted hybrid proteins), the route of immunization or the nature of the sequences immediately flanking the foreign epitope (3, 6–10).

In the present study, we compared directly for the first time the antibody responses induced against the same epitope expressed either at the bacteria cell surface, within LamB, or in the periplasm, within the MalE protein. Our results indicated that the cellular location (cell surface/periplasm) of the grafted epitope had a strong influence on the characteristics of the antibody responses and more particularly on the T cell dependence of these responses. In a second stage, we have studied

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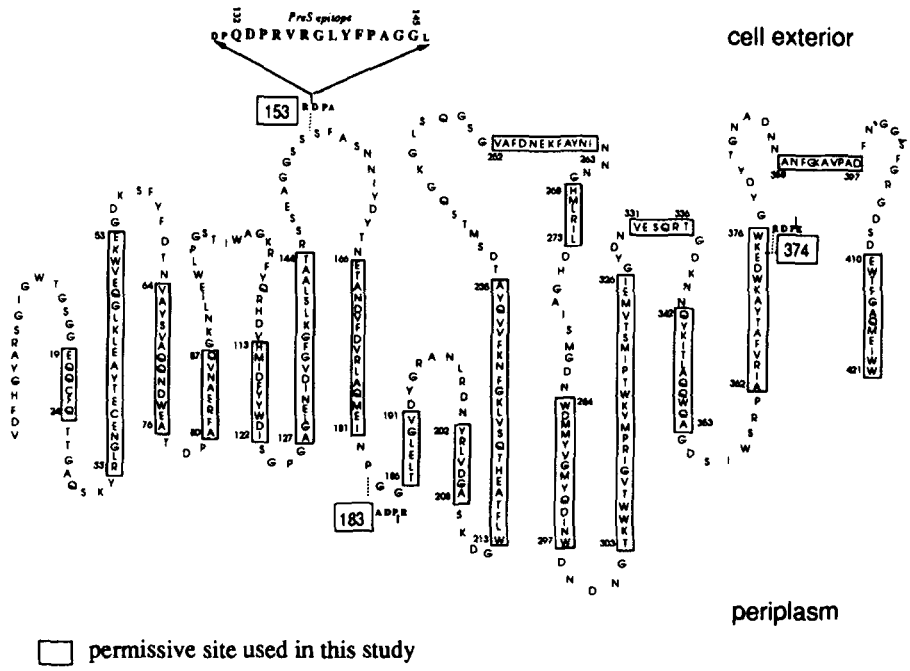

Figure 1. Location of the three permissive sites on the LamB folding model. The sequence of the mature LamB protein is represented in the *one letter amino acid code.* On this folding model, the LamB protein spans the membrane 16 times. The approximative width and position of the apolar part of the outer membrane are respectively represented by the *gray vertical arrow* (left of the model) and *horizontal bar.* The LamB regions predicted in β-configuration are presented in *white rectangles.* Permissive sites are named according to the residue position in the protein. Sites 153 and 374 result in expression of foreign sequences at the bacterial cell surface. Site 183 results in expression at the periplasmic face of the outer membrane. The sequence of the PreS epitope from HBV is indicated when inserted at position 153, yielding protein LamB-PreS(153). The supplementary residues due to the insertion of a *BamH1* linker are indicated in *small bold characters* (4).

the position effect of the foreign epitope with respect to the outer membrane when inserted in cell surface or periplasm exposed sites of LamB. We interpreted the results in terms of the degree of protrusion of the foreign epitope outside of the bacterial cell.

MATERIALS AND METHODS

Strains, media, and DNA techniques

LamB hybrids. Strain *E. coli* K12 pop 6510 (*thr, leu, ton B, thi, lacYI, dex5, metA, supE*) was the recipient for all the constructions. No LamB protein is detectable in this strain (11).

Plasmids pAJC264, pJAV183, and pAJC178, which correspond to the in-frame insertion of a 12-bp *Bam*H1 linker after amino acids 153, 183, and 374 of LamB, respectively, were previously described (2, 4) as well as the genetic insertions of a peptide comprizing residues 132–145 of the PreS2 region of HBV and a peptide comprizing residues 93–103 from VP1 capsid protein of poliovirus (also called C3 epitope) after amino acid 153 of LamB (2, 6). In this work, the PreS2 peptide was also inserted after amino acid residues 183 and 374 of LamB by the same procedure.

The four *lamB* hybrid genes were carried on plasmids derived from pAC1 (11). Their expression is under *ptac12* promotor control, and thus, inducible by isopropyl-b-D-thiogalactoside. The recombinant plasmids will be named pAJC264-VP1, pAJC264-preS, pJAV183-preS, and pAJC178-preS, and the corresponding hybrid proteins LamB-C3 or LamB-C3(153), LamB-PreS or LamB-PreS(153), LamB-PreS(183), LamB-PreS(374), respectively.

The hybrid proteins LamB-C3(153), LamB-PreS(153), LamB-PreS(183), and LamB-PreS(374) were present in similar amounts and conserved both functions of the LamB protein, i.e., phage receptor activity and dextrins utilization, indicating no major structural modifications of the proteins, and a normal localization in the outer membrane (data not shown). All this was to be expected from the properties of permissive sites (1, 5).

Detection of the foreign epitopes by specific mAb was assayed on bacterial extracts and semipurified proteins by Western blotting and on the native proteins by immunoprecipitation. Anti-preS mAb F376 was raised in mice immunized with native hepatitis B surface Ag recombinant particles (6). Anti-C3 mAb was raised in mice immunized with heat-inactivated poliovirus infectious particles (2). Both sera were used in Western blotting experiments at a dilution of 1/3000. In Western blotting experiments, denatured LamB-C3(153) and LamB-preS(153) hybrid proteins are specifically and clearly detected by mAb anti-C3, and mAb F376, respectively. We previously showed that, in both constructions, the foreign epitope is also accessible to the mAb on the surface of the native protein (2, 6).

MalE hybrids. All plasmids derived from plasmid pPD1 (12, 13). This plasmid carries the wild type *malE* gene under control of its own promotor. The expression of the *malE* gene is therefore sensitive to catabolic repression by glucose and induced by addition of maltose. Plasmids were expressed in strain ED9 carrying a nonpolar deletion in the chromosomic *malE* gene (*malEΔ444*) (12, 13). The genotype of ED9 is *E. coli* K12, F-*thiA relA araD139 ΔlacU169 rpsL ΔmalE444 recA1 srl::Tn10.*

Hybrid proteins, MalE:303-VP1:93–103 (abbreviated MalE-C3) and MalE:303-PreS2:132–145 (abbreviated MalE-PreS), had already been described (9). They are stably expressed in the periplasm of *E. coli* (about 60 μg/10^{10} bacteria). Hybrid MalE proteins were recognized in ELISA assays by specific anti-C3 and F376 mAb, and by polyclonal sera directed against the inserted epitopes.

For immunizations, bacteria were grown overnight at 30°C in LB broth medium (14) supplemented with 100 μg of ampicillin/ml. This culture was diluted 1/50 fold in 63 medium (14) supplemented with 100 μg ampicillin/ml, 1 mg thiamin/liter, 0.2% casamino acids and 0.4% glycerol. 0.2% maltose was added when A600 raised 0.3 and the culture continuated 1h30 to 2h30 (A600 = 1 to 1.4). Bacteria were collected by centrifugation and resuspended in PBS × 1.

Semipurification of LamB-PreS hybrid proteins

The hybrid proteins were prepared as described previously (15). Briefly, bacteria were grown at 37°C in liquid minimal medium supplemented with casamino acids and ampicillin, and with glucose as a carbon source. At OD_{600} = 0.4, isopropyl-b-D-thiogalactoside was added at a 10^{-3} M final concentration. At OD_{600} = 1, bacteria were collected by centrifugation, resuspended in lysis buffer (SDS 2%, glycerol 10%, MgC12 2 mM, Tris-HC1 10 mM, pH 7.4), and incubated 30 min at 60°C. The membrane fraction containing the peptidoglycan was pelleted by ultracentrifugation at 100,000 × *g* for 1 h. The LamB-PreS hybrid proteins were then solubilized from the pellet by an overnight incubation at 4°C in extraction buffer (Triton X100 2%, Tris-HC1 10 mM, pH 7.4). After centrifugation at 100,000 × *g* for 1 h, the hybrid proteins were recovered in the supernatant and concentrated in Triton X100 2% (0.5–1 ml for 500 ml of bacterial culture).

The amounts of proteins in the different extracts were estimated by comparing Coomassie blue band intensities from SDS-acrylamide gels with a standard preparation of wild-type LamB protein (kindly provided by Dr. D. Perrin, Institut Pasteur, Paris, France).

Detection of anti-LamB, anti-MalE, and antipeptide antibodies

The 6- to 8-wk-old female BALB/c mice (five to six per group) were from the animal colony of the Pasteur Institute (Paris, France). They were immunized as described in *Results* and bled various times after immunization.

Sera were analyzed by ELISA for the presence of anti-LamB or anti-MalE antibodies and for antibodies directed against the peptides as previously described (9). Wells of microtiter plates (Nunc, Nunc, Roskilde, Denmark) were coated with 500 ng of purified native wild-

type MalE or LamB proteins or of PreS peptide or with 150 ng of peptide (95–104). Peptide (95–104) (abbreviated C3) consisted of amino acid residues 95–104 of capsid protein VP1 of poliovirus type 1, flanked by additional Tyr-Gly-Cys-Gly residues at the N terminus and by Gly-Cys residues at the C terminus and was kindly given by Dr. S. van der Werf (Pasteur Institute). PreS peptide consisted of peptide (120–145) from the hepatitis B virus pre-S antigen (subtype ayw) and was synthesized by Neosystem (Strasbourg, France).

The negative control consisted of pooled normal mouse sera. Individual titers are expressed as the $\log_{10}$ of the maximal dilution giving an absorbance twice as high as the negative control diluted 1/100. Titers ± SE are calculated as the arithmetic mean of $\log_{10}$ titers.

In vivo elimination of CD4⁺ or CD8⁺ T cells

The L3T4 (CD4) specific rat anti-mouse hybridoma, GK1–5 was originally produced by Dialynas et al. (16). The CD8-specific rat anti-mouse hybridoma, H35.17.2 was obtained by Pierres et al. (17). Both mAb are of IgG2b isotype. Ascitic fluid preparations of mAb antibodies were used in all experiments. To obtain ascitic fluids, nude mice were pristane primed and injected with 10^6 hybridoma cells. Antibodies were prepared by ammonium sulfate precipitation. The quantity of protein was assessed by the measurement of optical density at 280 nm. To induce a specific long term in vivo depletion of $CD4^+$ or $CD8^+$ T cells, BALB/c mice were daily injected during 3 days with 300 μg of anti-CD4 or anti-CD8 mAb i.p. This treatment was shown in preliminary experiment to induce within 2 days an almost total depletion of the corresponding splenic T cell subset. The elimination of the $CD4^+$ T cell subset by anti CD4 mAb was accompanied by a complete suppression of antibody responses to T-independent Ag such as SRBC or keyhole limpet hemocyanin. The efficiency of the in vivo depletion of $CD8^+$ T cells has been controlled by the study of the capacity of treated mice to develop alloreactive cytotoxic T cells (18).

RESULTS

To study the influence of the location of a foreign peptide within a bacterial cell on its immunogenicity, we used two B cell antigenic determinants: the 132–145 PreS2 region of the hepatitis B virus (abbreviated here as PreS) (19) and the 93–103 neutralization epitope from poliovirus type 1 (abbreviated here as C3) (20, 21). We expressed these epitopes at different permissive sites of the LamB and MalE proteins and compared the antibody responses as described below.

Comparison of anti-PreS antibody responses induced by live recombinant bacteria expressing PreS epitope either at bacterial surface or in periplasm. We previously demonstrated that antibody responses against viral epitope(s) can be induced by immunization with live bacteria expressing these foreign epitopes either at the cell surface or in the periplasm. Therefore, the presentation at the cell surface of the foreign epitope is not a prerequisite to obtain an antibody response (6, 8, 9). However, we reasoned that the location of the inserted epitope in the bacterial cell could influence some characteristics of the induced antibody responses. Therefore, in the following experiment, we compared the kinetics and isotypes (IgM and IgG) of antibody responses induced against the PreS epitope inserted either in a cell surface exposed permissive site of LamB (site LamB153) or in the periplasmic MalE protein (site MalE303). Mice were immunized by i.p. injections of 2.10^8 live bacteria and control groups received recombinant bacteria expressing the C3 poliovirus epitope in the same LamB or MalE sites.

As shown in Figure 2, when the PreS epitope was inserted in the LamB protein, immunization with the recombinant bacteria induced an IgM anti-PreS antibody response that could be detected as early as 1 wk after the first injection of bacteria. This IgM antibody response further increased after a second injection and then

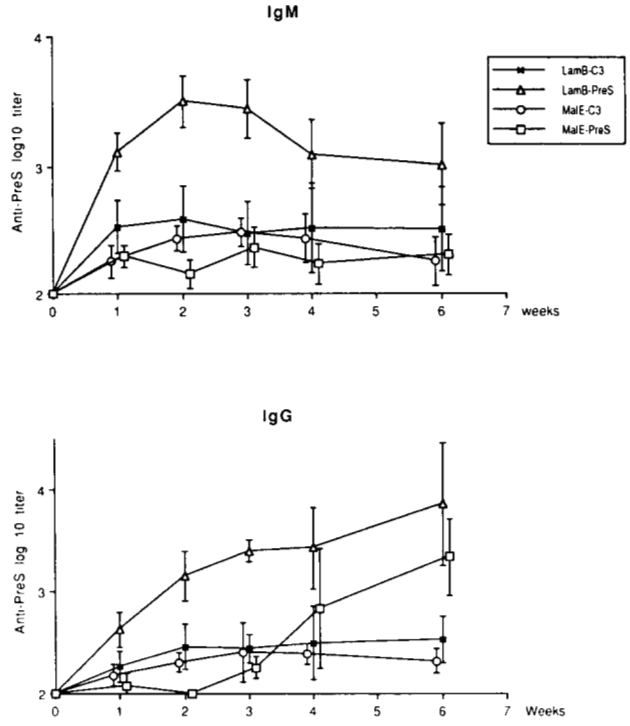

Figure 2. Comparison of anti-PreS antibody responses induced by live recombinant bacteria expressing the PreS epitope either at the bacterial surface or in the periplasm. On days 0, 7, 14, 21, and 35, BALB/c mice were injected i.p. with 2.10^8 live bacteria expressing LamB-C3(153), LamB-PreS(153), MalE-C3, or MalE-PreS. Mice were bled on days 6, 13, 20, 27, 42 and IgM (*upper panel*) and IgG (*lower panel*) anti-PreS antibody titers were determined on individual sera.

reached a plateau. In contrast, no IgM antibody response was detected after immunization with bacteria expressing the same PreS epitope in the MalE periplasmic protein, even after repeated administration of recombinant bacteria. It should be noticed that low but detectable levels of anti-PreS antibodies were induced by bacteria expressing the C3 poliovirus epitope, presumably due to polyclonal stimulation.

Immunization with bacteria expressing LamB-PreS also induced IgG anti-PreS antibody responses that could be detected in the week after the first injection of recombinant organisms and increased after subsequent administrations of the bacteria. In contrast, when the epitope was inserted in the MalE protein, the induction of IgG antibody responses required several injections of the recombinant bacteria but these responses reached maximum values comparable to those obtained after immunization with bacteria expressing LamB-PreS. It should be noticed that anti-PreS antibody responses obtained with bacteria expressing MalE-PreS were slightly inferior to those obtained after immunization with bacteria expressing LamB-PreS. However, these differences were not statistically significant and indeed opposite results were observed with the C3 epitope (Fig. 4).

Influence of in vivo elimination of $CD4^+$ T cells on anti-PreS antibody responses induced by live bacteria expressing either hybrid LamB-protein or hybrid MalE-protein. These results indicated that the location of the inserted epitope within the bacteria could affect some characteristics of the antibody response induced by the recombinant bacteria even though the total antibody response (IgM and IgG) measured after several injections of

the bacteria were comparable for the two carrier proteins (8, 9).

The antibody response induced by bacteria expressing MalE-PreS had the characteristics of a T cell-dependent antibody response (very low primary response followed by an IgG memory response) whereas the response induced by the bacteria expressing LamB-PreS presented some features of T cell-independent responses (rapid, intense, and prolonged IgM antibody response). Therefore, in the next experiment, we examined the possibility that the bacterial location of the grafted epitope influences the T cell dependence of the antibody response induced by these recombinant bacteria.

To address this question, mice were in vivo depleted of $CD4^+$ Th cells by injection of anti-CD4 mAb as previously described (18). We have previously established the experimental conditions to completely abolish the antibody response against T cell-dependent Ag such as SRBC or keyhole limpet hemocyanin (18).

As shown in Figure 3, mice treated with anti-CD4 mAb before and during immunization with bacteria expressing the LamB-PreS recombinant protein developed an IgM anti-PreS antibody response that was not different from that of untreated control mice. The IgG response of these mice was diminished but not suppressed by the anti-CD4 mAb treatment. Interestingly, the IgG responses that appeared one or two weeks after immunization were not affected by T cell depletion indicating the T cell independence of the early component of the IgG response.

Immunization with bacteria expressing the MalE-PreS recombinant protein only induced an IgG antipeptide antibody response. This response was totally abolished by in vivo treatment with the anti-CD4 mAb demonstrating the T cell dependency of these antibody responses.

Comparison of anti-C3 antibody responses induced by live recombinant bacteria expressing C3 epitope either at bacterial surface or in periplasm. To exclude that the previous results were specific for the PreS epitope, we performed similar experiments with recombinant bacteria expressing the C3 poliovirus neutralization epitope.

As shown in Figure 4, IgM antipeptide antibodies were induced only after immunization with live bacteria expressing hybrid LamB-C3 protein. The MalE-C3 protein did not stimulate IgM response but induced only a strong secondary IgG antibody response, starting 3 to 4 wk after the first injection. This response was totally abolished by anti-CD4 mAb treatment. The LamB-C3 protein also induced a moderate anti-C3 IgG antibody response that reached its maximum 1 to 2 wk after the beginning of immunization and did not increase after additional injections of bacteria. We did not examine the effect of anti-CD4 mAb treatment on the anti-C3 antibody response induced by hybrid LamB-C3 protein. However, the high IgM antipeptide response as compared to IgG response was in favour of the T cell independency of these responses.

The previous results indicated that distinct patterns of antipeptide antibody response can be induced by recombinant bacteria depending on the cellular location of the inserted epitope. Insertion in the LamB protein induced

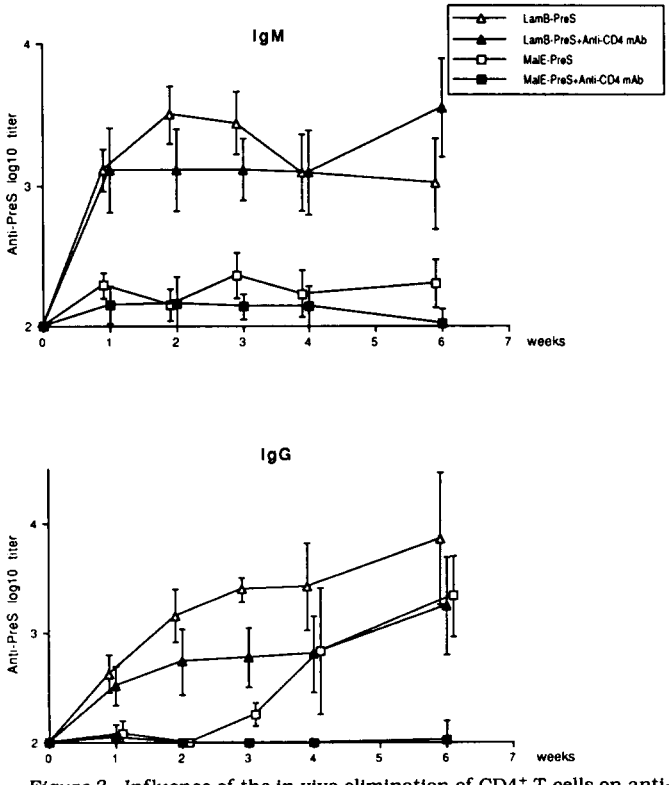

Figure 3. Influence of the in vivo elimination of $CD4^+$ T cells on anti-PreS antibody responses induced by live bacteria expressing either hybrid LamB-protein or hybrid MalE-protein. On days −1, 0, 1, 6, and 13, BALB/c mice were injected with anti-CD4 mAb (300 μg/mouse) or were left untreated. On days 0, 7, 14, 21, and 35, mice were injected i.p. with 2.10^8 live bacteria expressing either LamB-PreS(153) or MalE-PreS. Mice were bled on days 6, 13, 20, 27, 42 and IgM and IgG anti-PreS antibody titers were determined on individual sera.

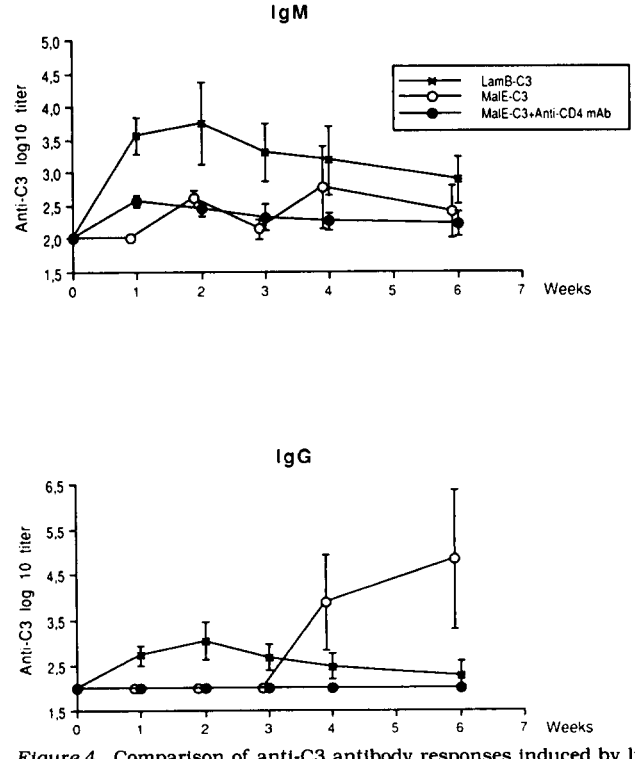

Figure 4. Comparison of anti-C3 antibody responses induced by live recombinant bacteria expressing the C3 epitope either at the bacterial surface or in the periplasm. On days −1, 0, +1, 7, 14, and 21, BALB/c mice were injected with anti-CD4 mAb (300 μg/mouse) or were left untreated. On days 0, 7, 14, 21, and 35, mice were immunized with 2.10^8 live bacteria expressing either LamB-C3(153) or MalE-C3. Mice were bled on days 6, 13, 20, 27, and 42 and IgM and IgG anti-C3 antibody titers were determined on individual sera.

mainly a T cell-independent response whereas the antibody responses induced by MalE hybrid proteins were strictly T cell dependent.

We next tried to see if these findings obtained with defined epitopes could be extended to the antibody responses directed against the whole LamB and MalE carrier proteins. We therefore examined the anti-LamB and anti-MalE IgM and IgG antibody responses in control and anti-CD4 mAb-treated mice. The bacteria expressing LamB-C3 induced an IgM and IgG anti-LamB antibody response in control animals. The anti-CD4 mAb treatment did not significantly modify the IgM antibody response and reduced only partially the IgG response. Bacteria expressing MalE-C3 induced almost only IgG anti-MalE antibodies and this response was totally abolished by $CD4^+$ T cell depletion (data not shown). These data indicate that the characteristics of antibody responses directed against inserted foreign epitopes reproduced those of the carrier proteins.

At this stage, it could be proposed that the marked qualitative difference in antibody responses induced by LamB and MalE proteins was due either to intrinsic differences between the two proteins or to their different cellular location (surface vs periplasm). To address this question, we examined the antibody response directed against hybrid LamB-PreS protein solubilized from the outer membrane and semipurified.

As shown in Figure 5, in contrast to what was observed after immunization with live bacteria, the immunization with solubilized semipurified LamB-PreS protein induced mainly an IgG response both against the inserted PreS epitope and the LamB protein itself. Moreover, this response was totally abolished in mice treated with anti-CD4 mAb. Therefore, the T cell independence of LamB responses was due to the cellular location of LamB and not to its intrinsic properties.

Influence of position of foreign peptide regarding bacterial membrane. We next wanted to see if the exact position of the foreign epitope within the LamB protein expressed on the outer membrane could influence the antibody response. For this we compared the immunogenicity of the PreS epitope inserted at site 153 and at two additional permissive sites of LamB: one site (site LamB374) is located at the cell surface-like site 153, and the other one (site LamB183) is exposed to the periplasm (2, 4) (Fig. 1).

Upon immunization of mice with live recombinant bacteria significant anti-PreS antibody responses were only obtained when the foreign epitope was inserted at site 153 whereas the anti-LamB responses were similar in the three cases (Fig. 6 and legends). The very weak anti-PreS antibody responses obtained with bacteria expressing the PreS epitope at site 183 and 374 were indeed not significantly different from those obtained against the unrelated C3 peptide (data not shown).

These results indicated that, in intact cells, the immunogenicity of the inserted epitope was influenced by the insertion site on the carrier protein. These differences in immunogenicity could be due either to modifications in the location of the epitope within the LamB protein itself (intramolecular location), or to other modifications in the context of the foreign epitope due to its position in the bacterial cell (cellular location).

To distinguish between these two hypotheses, we compared the immunogenicity of the three recombinant LamB-PreS proteins after extraction from the outer membrane and semipurification. In the three cases, the anti-PreS responses were similarly high (Fig. 7). Therefore, the striking differences in immunogenicity between the three sites seen with intact bacteria cannot be accounted for by differences due to the intramolecular location of the epitope on the purified proteins.

A simple hypothesis would be that the position with respect to the outer membrane is important so that, for example, epitopes expressed in LamB at the internal face of the outer membrane would not elicit antibodies. This would account for the fact that no response was obtained when the epitope was at site 183. However, the absence of response in the case of site 374 that is exposed at the cell surface is not readily explained by this hypothesis.

At this stage, it is useful to recall that in intact cells, the C3 epitope was more easily detected at site 153 than at site 374 for all assays (RIA, immunoelectron microscopy) (2, 4). This suggested that site 153 protrudes more on the outside of the cell than site 374. This hypothesis is also supported by results obtained on intact cells expressing the wild type LamB protein, with polyclonal antibody directed toward synthetic peptides encompassing sites 153 and 374 (22). It also accounts for the fact that, when the PreS peptide was expressed at the same sites, it could only be detected in intact cells at site 153 when a specific mAb F376 was used in the assay (6).

Thus, the results obtained with the PreS epitope expressed at sites 153, 183, and 374 is compatible with the idea that to obtain an antibody response with intact bacteria, the epitope has to be protruding sufficiently to the outside of the outer membrane.

Finally, it is worth noticing that all three LamB-PreS

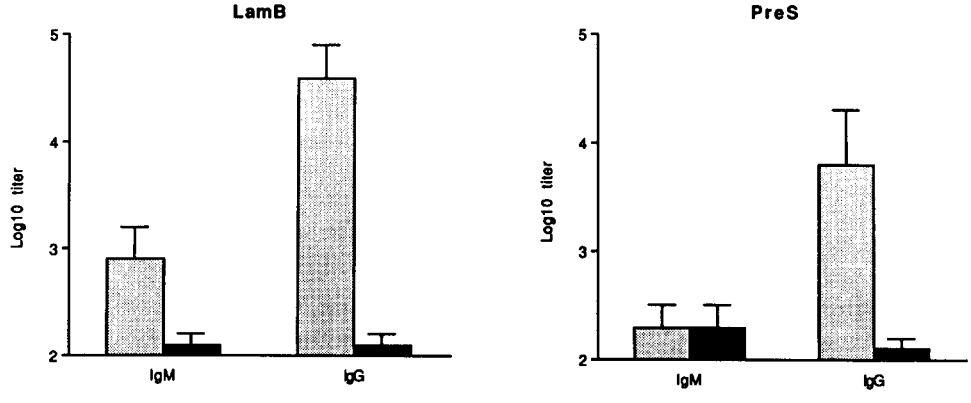

Figure 5. T cell dependance of antibody responses induced by purified hybrid LamB proteins. On days −1, 0, 1, 20, 21, 22, BALB/c mice were injected with anti-CD4 mAb (300 μg/mouse) (■) or were left untreated (□). On days 0 and 21, all mice were immunized with 25 μg of purified hybrid LamB-PreS(153) protein together with 1 mg $AL(OH)_3$. On day 31, mice were bled and antibody titers against LamB and preS peptide (120–145) were determined on individual sera.

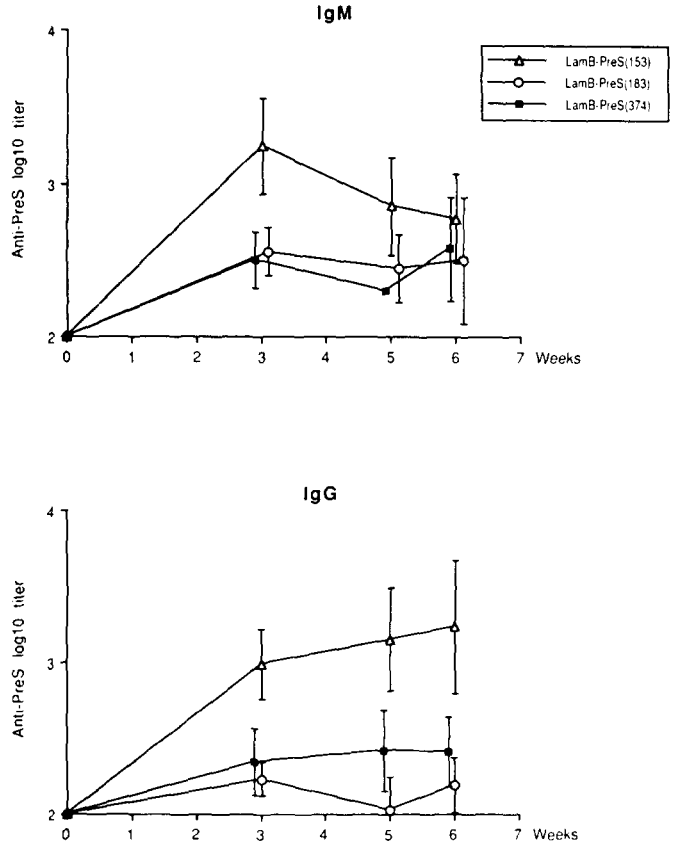

Figure 6. Induction of anti-PreS antibody responses by recombinant bacteria expressing the PreS epitope at the surface of the bacteria: influence of the position of the foreign peptide with regard to the bacterial membrane. On days 0, 7, 14, 21, and 35, BALB/c mice were injected with 2.10^8 live bacteria expressing LamB-PreS (153), LamB-PreS (183), or LamB-PreS (374). On days 20, 34, and 42, mice were bled and IgM and IgG anti-PreS antibody titers were determined on individual sera. On day 42, the anti-LamB antibody titers were 5.0 ± 0.2 in the three groups.

purified hybrid proteins induced mainly IgG anti-PreS antibodies and only weak IgM responses. This supports the idea that intact bacteria expressing the LamB-153-PreS protein induce both IgG and IgM anti-PreS antibodies, primarily because of the cellular location of the epitope.

DISCUSSION

In the present study, we identified two major pathways by which bacterial epitopes can stimulate specific antibody responses. The first one is mediated by direct activation of B cells by cell-surface Ag. It does not require T cell help but requires that the epitope be well exposed at the surface of the bacterial cell. The second one is T cell dependent and concerns Ag that can be released from the bacteria in a soluble form. This study also provided the first evidence that the position of a B cell epitope within a molecule may not influence its immunogenicity, at least in the cases studied.

Immunization of mice with live bacteria expressing the PreS or C3 B cell epitopes at the cell surface induced T cell-independent antibody responses that were characterized by the rapid induction of IgM responses. These responses resisted anti-CD4 mAb treatment. The IgM antibody response was followed by the appearance of IgG-specific antibodies that were partially T cell independent. The T cell independence of anti-PreS antibody responses required that the epitope be expressed at the cell surface because immunization with purified LamB proteins induced typical T cell-dependent responses. The absence of a T cell requirement for antibody responses against epitopes located at the *E. coli* cell surface could indicate that B cells were stimulated by direct contact between their surface Ig and the epitopes expressed by the bacteria. This hypothesis is supported by our data showing that the highest anti-PreS antibody responses were induced with intact bacteria when the PreS epitope was inserted at site 153, which corresponds to a well exposed loop of the LamB protein. The degree of protrusion of the foreign epitope on the outside of the bacterial cell could be expected to have a major influence on the recognition of the epitope by surface Ig of specific B cells.

It is well established that the differenciation of B cells into secreting plasmocytes requires at least two signals. The first one is given by the Ag and the second one occurs through direct B and T cells interaction or via lymphokine signaling. It could be suggested that bacterial polyclonal B cell activators such as LPS will provide this second nonspecific signal to B cells interacting with the gram-negative *E. coli* cell surface. It has already been shown that Ag coupled to LPS become T independent (23). Alternatively, the T cell-independent activation of B cells could also occur through cross-linking of their Ig by the repetitive epitopes at the bacterial cell surface. Indeed, the nucleocapsid of hepatitis B virus that is a multimeric 27-nm particle composed of multiple copies of a single polypeptide has been shown to be both a T cell-independent and a T cell-dependent Ag (24). Inasmuch as hepatitis B virus does not possess direct polyclonal properties, it is likely that the T cell independence of nucleocapsid of hepatitis B virus is due to the high epitope density of the particles. Interestingly, it has also recently been shown that covalent surface linkage of Ag at the surface of

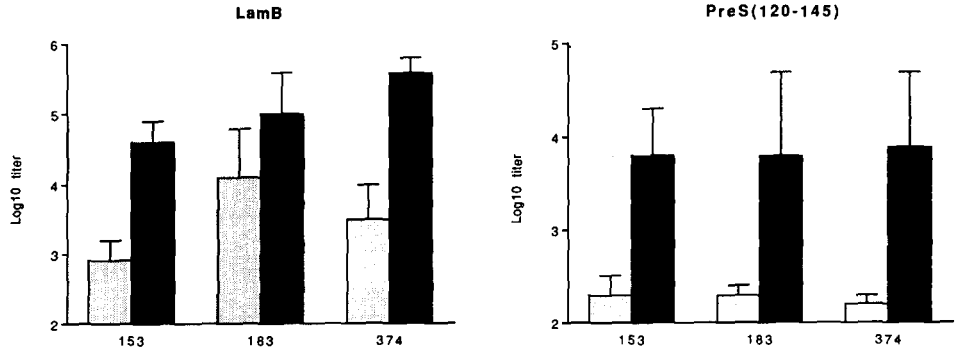

Figure 7. Induction of anti-PreS antibody responses by purified hybrid LamB proteins expressing the PreS epitopes in different sites. On days 0 and 21, BALB/c mice were immunized with 25 μg of purified hybrid LamB proteins prepared from bacteria expressing LamB:153-PreS(153) or LamB:183-PreS(183) or LamB-PreS(374) together with 1 mg $AL(OH)_3$. On day 31, mice were bled and IgM (□) and IgG (■) antibody titers against LamB and preS2 peptide (120–145) were determined on individual sera.

liposomes induces an important production of IgM (25). In contrast, encapsulation leads predominantly, although not exclusively to the production of IgG.

When the foreign epitope was inserted into the MalE protein and therefore expressed in a soluble form within the periplasm (e.g., inside the bacterial particle), the antibody responses were strictly T cell dependent. The cognate interaction between T and B cells has been shown to be mediated through Ag presentation by the B cell. We have recently shown that B cells cannot present particulate Ag such as keyhole limpet hemocyanin linked to polyacrolein microspheres (A. Galelli and C. Leclerc, manuscript in preparation). These beads were 0.25 to 1 μm in diameter, which is approximately the size of bacteria. This indicates that bacterial Ag could not be presented directly by B cells and would be presented only after lysis of the bacteria and release under a soluble form. We have previously shown that live bacteria expressing MalE hybrid proteins are not immunogenic when given s.c., but that a response was induced when the MalE hybrid protein was released under a soluble form from the bacterial cell (9). For Ag expressed inside the bacteria, the induction of antibody responses would therefore require their release into a soluble form before the interaction of the bacteria with APC.

Thus, the bacterial location of an epitope determines the T cell dependence of the antibody response: Ag expressed at the cell surface are T independent whereas internal Ag are T dependent. To become immunogenic, internal Ag would have to be released into a soluble form by lysis of the bacteria. It should be interesting in this respect to determine if epitopes inserted in cytoplasmic proteins give patterns of responses that are similar to those obtained by insertion in the periplasmic MalE protein. Obviously, some external T-independent Ag would also induce T-dependent responses if they could be released into soluble form. Bacterial Ag that are not expressed at the bacterial surface or cannot be released into soluble form may not be immunogenic. Moreover, because our data show that the immunogenicity of grafted epitopes are dictated by the carrier proteins, it should be possible to orient the responses to a foreign epitope by appropriate selection of the recipient protein. In terms of vaccine development, expression at the bacterial surface would induce rapid antibody response and would induce a protection within few days even in immunodeficient individuals lacking Th function. However, the memory response induced by these epitopes located at the surface could be deficient as shown for most T cell-independent Ag (26). In contrast, induction of antibody responses by internally expressed epitopes would require several weeks but these epitopes would be able to induce efficient B cell memory. Recombinant vaccines expressing a foreign epitope in both locations would potentially be able to stimulate rapid T-independent antibody responses followed by a long lasting memory.

Another striking result of this study is that the immunogenicity of the PreS epitope was similarly high at the three different sites of the solubilized hybrid proteins. To our knowledge, this is the first time that the immunogenicity of the same peptide has been compared at different sites of a protein and it will be interesting to see if it is a general observation. It is important to recall that the insertion sites used here have two important properties. First, they are permissive sites corresponding to regions of the protein that are flexible enough to accommodate a foreign sequence without a strong effect on the protein structure. Such sites correspond most of the time to regions located at the surface of proteins (7). Second, they display the foreign epitope in such a way that it can be detected with a specific mAb. Thus in all three cases, the epitope was accessible at the protein surface and free to adopt conformations able to react with the mAb.

It is not known at present whether these two properties are general conditions for good immunogenicity. It is interesting to note that they are the basis for some predictions of B epitopes (27). Further work with foreign epitopes expressed at other permissive sites of LamB (including at those where the peptide is not detected by mAb (4)) and at different permissive sites of other proteins such as the periplasmic maltose binding protein (5, 9) should provide more information pertinent to these questions.

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