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**ABSTRACT**

*Staphuloccocus aureus* enterotoxin A (SEA) is a toxin involved in numerous food poisoning cases. To develop a detection test specific for, different approaches have been envisaged to obtain highest antibody titer sera against a peptide sequence from this protein. The present work compares the properties of antibodies raised against the peptide epitope by classical and multiple antigen peptide (MAP) system approaches. Different immunization protocols were used: BALB/c mice were immunized either with the peptide or the MAP alone in Freund's adjuvant, co-immunized with the keyhole limpet hemocyanin (KLH) protein or, with the peptide, conjugated to this carrier KLH protein.

The extent of reaction of the antibodies to the MAP construct with the parent protein was found to be significantly less than the antibodies raised against the monomeric peptide co-immunized with or conjugated to a carrier protein but more that the antibodies raised against the peptide alone. Inversely, co-immunization of the MAP with the KLH was not able to raise the immune response as it was observed with the monomeric peptide. The results suggest that, for the epitope chosen here, MAP constructs were not the most effective option to induce sera with high levels of antibodies that react with the native protein.

## 1. Introduction

Among the wide variety of exoproteins produced by *Staphylococcus aureus* that contribute to cause disease in mammalian hosts (Dinges et al., 2000), the *S. aureus* enterotoxin A (SEA) is of particular importance since 70% of all identified causes of staphylococcal food poisoning are due to SEA (Bergdoll, 1979). To elaborate a detection test specific for this protein with antibodies that discriminate SEA from other enterotoxins, a peptide specific of this protein have been selected.

The peptides of about 16-17 amino acids have a molecular weight in the range of 1.7-2 kDa. These molecular weights are generally not sufficient to evoke an immune response. For this reason, the synthetic peptides must be converted to high-molecular weight products to elicit an antibody response. Branched peptides such as Multiple Antigen Peptides (MAPs) (Tam, 1988; Posnett et al., 1988) have been extensively tested to use single epitope to produce antibodies. MAP consists of a core of branching lysine residues to which peptides epitopes of interest are covalently attached. The resulting polypeptide reaches sufficient molecular weight to stimulate the immune system and could be considered as a small protein. This was an alternative to the classical carrier protein approach consisting to conjugate the peptide to a carrier protein like bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). In addition to obtain a peptide-containing chimeric molecule with high molecular weight, these carrier proteins bring also T-cell epitopes, leading to a proliferation of T-helper cells and an effective stimulation of the immune system.

MAP peptides have been used for various immunological applications but mainly for antibodies production and stimulation of the immune system. In this case, they are presented as an efficient alternative to peptide-protein conjugates (Tam, 1988) with a great potential as immunogens or synthetic vaccines. They have successfully been used for experimental vaccination against

various infectious diseases such as malaria (Edelman et al., 2003; Joshi et al., 2001; Nardin et al., 1995), schistosomiasis (El Ridi et al., 2004) or HIV-1 (Misumi et al., 2001; Nardelli and Tam, 1993).

The increased molecular weight and repeating peptide structure of the MAP has been shown to result in improved B-cell recognition and antibody specificity (Briand et al., 1992). MAPs allow also various applications like polyepitopic constructions (Smith et al., 2005), that allow association of peptide of interest with T-cell epitope-containing peptides. Whatever is the case, when comparisons are realized between the peptide either as a monomeric or as a MAP form, only with an adjuvant but without any other protein, the last construction is fully the most efficient to generate the best antibody production (Tam, 1988; Tam and Zavala, 1989; McLean et al., 1991; Wang et al., 1991; Francis et al., 1991; Marguerite et al., 1992; Baleux and Dubois, 1992; Marussig et al., 1997; Farris et al., 1999)

However, conclusions are sometimes guarded about efficiency of MAP peptides as good promoters of immune response and some experiments underline the limits of this technique (Briand et al., 1992; Mahale et al., 1996).

In order to estimate and possibly to enhance the effectiveness of antibody production with a multiple antigen peptide, comparisons to immunizations with the peptide without or with the presence of a carrier protein, with or without conjugation, have been realized. Raised antisera in mice were compared in ELISA against the SEA native parent protein or against the peptides (monomeric or MAP).

The administration of the peptide conjugated to KLH induced the best antibody production. Co-immunization of the monomeric peptide with the KLH protein induced a strong increase in antibody production compared with immunization by peptide alone which was the

least potent in the induction of such responses. On the other hand, co-immunization of the MAP peptide with KLH did not increase the antibody response.

## 2. Materials and Methods

### *2.1. Reagents*

The choice of the peptide sequence has been done in order to maximize the probabilities to obtain a good antibody production. In this purpose, well-exposed avoiding complex secondary structures, hydrophilic sequences have been tested. The peptide sequence in this study has been chosen among four other peptides of the SEA protein because it gave the best immune response in preliminary tests on mice. The peptide sequence (TEPSVNYDLFGAQGQYSN) represented the amino acids 190 to 207 of the SEA protein. Peptides were synthesized by the society GenScript corporation (Piscataway, NJ, USA). The monomeric peptide was resupended at 5 mg/ml in deionized water. The same amino acid sequence was synthesized in a multiple antigen peptides (MAPs) form as tetravalent (tetrabranched) construct using FMOC chemistry on an automatic synthesizer according to the manufacturer's protocol. They were then purified by reverse-phase high-performance liquid chromatography (RP-HPLC) and were provided lyophilized and stored desiccated until use. Mass Spectrometry (MS) analysis confirmed the expected molecular mass expected. Resuspension was done at the concentration of 5 mg/ml in deionized water. All experiments reported here were conducted using the same lots of purified peptides.

A second peptide with an added C-terminal cysteine was synthesized allowing conjugation, via the sulfhydryl group of this amino acid, to the amino group of lysine residues on the keyole limpet hemocyanin (KLH) (Sigma, St. Louis, MO, USA) using MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester) coupling method. This monomeric peptide conjugation to KLH was effected in order to keep the same peptide orientation as in the MAP.

Highly purified SEA (purity > than 95 %) was obtained from Toxin Technology (Sarasota, FL, USA) and was redisolved in deionized water to a 1 mg/ml concentration.

*2.2. Animals and immunization*

Adult female BALB/c mice (Janvier, Le Genest, France) were divided in groups of 6 mice and immunized with the different formulation of the epitope as a MAP peptide or as a single peptide, either with or without adjunction of KLH as described below and recapitulated in Table 1. For immunization, peptides and MAP were diluted in phosphate-buffered saline (PBS). In each case, the immunizations result in the injection of equal moles of the specific epitope and of KLH when it is present. Two groups were immunized with 25 µg of MAP peptide (corresponding to 10.6 nM of individual peptide sequence) without (group MAP) or with the presence of 125 µg KLH (group MAP + KLH). Two other groups were immunized with 25 µg of the classical peptide (corresponding to 11.7 nM of peptide) without (group Pep) or with the presence of 125 µg KLH (group Pep + KLH). A last group was immunized with 25 µg of the peptide conjugated to the same quantity of KLH as previously (group Pep-KLH). Control mice were immunized with PBS incorporated in Freund's adjuvant for one group and the other group with KLH in Freund's adjuvant.

Mice were immunized at multiple sites by subcutaneously way, four times, with about one month of interval (D0, D28, D57 and D87). Antigens diluted in PBS were emulsified 1:1 in complete Freund's adjuvant (CFA, containing the H37Ra, ATCC 25177 *M. tuberculosis* strain) for the first immunization (D0) or incomplete Freund's adjuvant (IFA) for the three following immunizations (D28, D57 and D87). Blood samples were collected two weeks after the first immunization (D14) or one week after the last immunization for the boosts (D35, D64 and D94) from the retro-orbital

plexus and sera were stored at −20°C until use. All animal experiments were done in accordance with institutional and national ethical guidelines.

*2.3. Enzyme-linked immunosorbent assay (ELISA)*

The antibodies were screened for binding to the monomeric peptide, the MAP and the native protein using the ELISA method. Maxisorp polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0,5 µg/ml of highly purified SEA protein or with 1 µg/ml of monomeric peptide or MAP in 100 µl of carbonate–bicarbonate buffer (0.1 M, pH 9.6). After a wash with phosphate-buffered saline–Tween 20 (PBS–T) (0.05%, v/v), plates were blocked for one hour at 37°C with PBS–T containing 0.5% bovine serum albumin (PBS–T–BSA). Then serially diluted sera in the same buffer were added to the wells. After 90 min of incubation at 37°C and four washes with PBS-T, goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA alkaline phosphatase-conjugated (Southern biotechnology, Birmingham, USA) were added and the plates were incubated for another 1 h 30 at 37°C. After four washes, 100 µl of 1 mg/ml of *p*-nitrophenylphosphate (Sigma) in diethanolamine buffer (pH 9.8) was added, and then plates were read at 405 nm with a Titertek Multiscan instrument (Skatron, Oslo, Norway).

*2.4. Statistical analysis*

Significant values were generated using the student t-test. A P value $<0.05$ was considered to be statistically significant.

## 3. RESULTS

### *3.1. Antibody titer*

Titers of MAP or relative peptide-induced antibodies reactive either with the native SEA protein (Fig. 1), the peptide or the MAP (Fig. 2) were analyzed by ELISA. The results presented here are those obtained after four immunizations but those observed after the second and third immunizations (data not shown) were equivalent in terms of quality.

High titres of antibodies to the parent SEA protein were obtained following the immunization of mice either with the peptide conjugated to KLH or with unconjucated monomeric peptide and KLH (Fig. 1). The titres generated by the MAP formulations were fully weaker either with or without KLH presence. Mice immunized by MAP without KLH presented even better antibody sera titres than mice co-immunized with MAP and KLH. On the contrary response generated by the peptide alone was negligible in comparison with the one generated with peptide KLH–added and with control sera.

Anti-monomeric peptide antibodies generated in presence of KLH bound peptide and MAP almost to the same extent in the ELISA system used (Fig. 2A and B). Conversely, antisera to the MAP construct were more efficient in MAP ELISA system than with monomeric peptide coated ELISA plates. Low titres of antibodies from peptide alone-immunized mice sera were observed in both cases.

After the first immunization (data not shown), only peptide KLH-conjugated immunized mice sera had notable antibody titres when linear monomeric peptide or MAP, but not SEA, were used in ELISA coating.

*3.2. Antibody isotype and IgG subclass analysis*

To further characterize the antibody response, IgG subclass, IgM and IgA reactivities either with, the native *S. aureus* SEA protein, the peptide or the MAP were examinated by ELISA after the first (Fig. 3) and the fourth immunization (Fig. 4). The optical densities (ODs) obtained for the four IgG subclasses, IgA, and IgM, were measured with sera diluted at 1/625 for the first immunization (Fig. 3) and 1/2500 for the fourth immunization (Fig. 4). The results obtained with the immunoglobulin isotypes are in agreement with those obtained previously in titres study.

The antibody response depended on the formulation of the peptide used during immunization. After the first immunisation (Fig. 3), when the monomeric peptide or the MAP were used for coating (Fig. 3B and C), there was mainly, as expected, weak IgM reponses in mice immunized with the MAP, MAP + KLH, peptide and peptide + KLH. On the contrary, mice immunized with peptide conjugated to KLH already presented a good IgG response, more particularly IgG1 (O.D. = 0.77 vs. peptide and O.D. = 1.89 vs MAP). When SEA was used for coating (Fig. 3A), IgG were not able to recognize the native protein and only a weak IgM response (O.D. = 0.12) was observed in this Pep-KLH group.

After the fourth (third boost) immunizations (Fig. 4), stronger responses were observed in all immunized groups, except group immunized with the monomeric peptide alone. The group immunized with the peptide conjugated to KLH gave the strongest antibody response for IgG1 (O.D. = 3.70), IgG2a (O.D. = 1.59) and IgG2b (O.D. = 2.20) when SEA was used in coating (Fig. 4A). It is interesting to note that this last IgG2b subclass was strongly reduced ($P<0.0001$) when immunization was done with the peptide and KLH unconjugated, while the two other subclasses (IgG1 and IgG2a) presented no significant difference in intensity response. Peptide immunized group did not show any significant antibody response. In comparison to Pep+KLH immunization,

the MAP+KLH group response was very weak. For example the strongest immunoglobulin, IgG1, presented a 0.48 O.D. value. The MAP alone immunized group even showed better IgG2a ($P<0.05$) and IgG2b ($P<0.01$) responses than those of this MAP+KLH group.

When the monomeric peptide (Fig. 4B) or the MAP (Fig. 4C) were used in ELISA coating, the results were relatively equivalent regarding the Ig isotype intensity order. As previously, the major fact that could be noticed was the IgG2b response reduction between peptide KLH-conjugated and unconjugated KLH-added immunized mice ($P<0.002$). In addition, some minor observations could be revealed. For example, augmentation of specific IgG3 isotype could be detected in sera from mice when KLH was added to the MAP ($P<0.001$) but only when the monomeric peptide was used for coating (Fig. 4B). With MAP coating (Fig. 4C), there was a weak IgM response with the peptide alone immunization that was not as stronger when using peptide coating ($P<0.05$). (Fig. 4B). Logically, the intensity observed in MAP immunized groups (MAP and MAP+KLH) were globally more important with the MAP coating than with peptide coating.

The results after the second and the third immunizations (data not shown) were equivalent in intensity and antibody isotypes profiles to those observed after the fourth immunization except few minor variations. The results observed in the IgG subclasses and Ig isotypes studies (Fig. 3 and 4) were in agreement with the results reported for titres (Fig. 1 and 2).

Globally, an overall view of the results showed that humoral immune response stimulatory potency of peptide plus KLH unconjugated formulation was significantly more efficient than the MAP plus KLH one. This was particularly observed in the parent SEA protein recognition.

## 4. Discussion

The main aim in the development of peptide-based antibodies production is to obtain antibodies that recognize the whole original protein. In this purpose, short peptide fragments containing the most potent antigenic determinants can be selected according to physicochemical structural properties such as surface accessibility, hydrophilicity or segmental mobility. However, due to their small size, peptides need to be conjugated to a carrier protein to generate an effective immune response.

Branched immunogens have been developed to bypass the requirement of a carrier and reported to be more stable than linear peptide (Fitzmaurice et al., 1996) and to induce immune response against the peptide branches but not the matrix core, i.e. the polylysine (Posnett et al., 1988). According to Fitzmaurice et al. (2000), peptides branches are presented to T-cells by professional antigen-presenting cells more efficiently than linearly arranged determinants.

Although MAPs are valuable tools for generating specific antibodies, they are often poor immunogens due to their lack of relevant T-cell epitope. To avoid the construction of a chimeric fusion molecule containing an added foreign irrelevant T-cell epitope to the proteic sequence of interest, the goal of this study is to test if co-immunization of the MAP with a complex protein like KLH, is able to improve the immune response.

The MAP construct was immunogenic as shown by the fact that mice elicited high antipeptide antibodies response. Antipeptide antibodies to the MAP construct bound SEA as well as MAP or peptide. As reported elsewhere, when only Freund's adjuvant is used, comparison of immunization between MAP and relative monomeric peptide show without any doubt that MAP are widely more efficient than peptide in specific antibody generation (Tam, 1988; Tam and Zavala, 1989; McLean et al., 1991; Wang et al., 1991; Francis et al., 1991; Marguerite et al., 1992; Baleux and Dubois, 1992; Marussig et al., 1997). This is also the case here. However, the

extent of binding was found to be significantly less as compared to that of antisera elicited against the peptide-KLH conjugate or peptide co-injected with KLH.

In fact, although the MAP method has been shown to result often in high antibody titers against the peptide, the antibodies seem to recognize less often the protein (McLean et al, 1991; Arnon and Van Regenmortel, 1992; Darcy et al., 1992; Mahale et al., 1996). One possible explanation of this behavior could be that the polylysine core stabilizes the peptide in a certain conformation and that a less flexible peptide results from this for antibody production.

Concerning the monomeric peptide, conjugation to a carrier protein is not absolutely necessary to obtain serum with high titer level of antibodies but the simple association, via a co-injection, of the peptide with a protein usually used for conjugation, like KLH, is sufficient in the case studied here. This approach of co-immunization could avoid some problems encountered when peptides are conjugated to a protein carrier, resulting often in complex mixture of products. For example, the carrier protein may modify some determinants and the structure of the peptide. However, it does not seem to occur here. Indeed, the best response and parent protein recognition remain when this conjugation is present. In addition, it is interesting to note that conjugation to KLH is a strong inductor of IgG2b in serum.

The surprise comes from the fact that when the MAP is co-injected with KLH, there is not such a huge humoral immune response as those observed for the peptide. On the contrary, there is even a slight decrease in the SEA and monomeric peptide sera recognition capacity in comparison to mice immunized with MAP alone. To explain this, we can refer to works demonstrating that the MAP structure induces T-cell response by itself (Olszewska et al., 2000; Farris et al., 1999; Costa et al, 2003), so, the addition of a protein like KLH is useless to generate T-help.

In addition, it can also be noted that results are similar when the peptide is used with its monomeric or MAP formulation for coating the ELISA plates, even if the MAP coating or

presentation of the peptidic sequence via the MAP is slightly more efficient, as shown by Tam and Zavala (1989) or Briand et al (1992). So, it can be estimated that binding properties to plastic solid surface are relatively the same for the MAP and their monomeric counterparts. As observed in the experiments of Tam and Zavala (1989) for example, this is not always the case and depends certainly on hydrophobic properties of the peptide. A poly-lysine pre-treatment of the plate could increase the binding of the peptide on the bottom of the plate. Furthermore, it also indicates that antibodies generated against the central lysine core are negligible, in agreement with results obtained in other studies (Posnett et al., 1988).

In conclusion, these results demonstrate that the form of the antigen and its association with a protein can profoundly influence the nature and magnitude of the immune response. To obtain good levels of antibody response, the classic method, the simple peptide associated with another protein (with but also without conjugation), remains the best method in the case presented here. The study of the antibody responses showed that the MAP formulations were not able to induce a good antibody response. The optimal response resulted from the classical method using a carrier protein conjugated monomeric peptide.

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**Figure legends**

**Figure 1:** Reactivities of antisera with SEA by ELISA.

SEA (1 µg/ml) was immobilized to the microtiter plate and incubated with serial dilutions of the antisera obtained after four immunizations with the different formulations of the peptidic sequence. Results are presented as the mean optical densities (± standard errors) for each group of six immunized mice.

**Figure 2:** Reactivities by ELISA of antisera to monomeric peptide and MAP.

Monomeric peptide (A) or MAP (B), both at 0.5 µg/ml were immobilized to the microtiter plate and incubated with serial dilutions of the antisera obtained after four immunizations with the different formulations of the peptidic sequence. Results are presented as the mean optical densities (± standard errors) for each group of six immunized mice.

**Figure 3:** Immunoglobulin M, A isotype and Immunoglobulin G subclass analysis after the first immunization. Sera obtained from immunized BALB/c mice two weeks after the first immunization were tested in ELISA diluted at 1/625 for IgG1, IgG2a, IgG2b, IgG3, IgM and IgA against SEA (A), monomeric peptide (B) or MAP (C). The results are presented as the mean optical densities (± standard errors) of the different isotypes for each group of six immunized mice.

**Figure 4:** Immunoglobulin M, A isotype and Immunoglobulin G subclass analysis after four immunizations. Sera obtained from immunized BALB/c mice one week after the fourth

immunization were tested in ELISA diluted at 1/2500 for IgG1, IgG2a, IgG2b, IgG3, IgM and IgA against SEA (A), monomeric peptide (B) or MAP (C). The results are presented as the mean optical densities (± standard errors) of the different isotypes for each group of six immunized mice.

**List of the abbreviations:**

ELISA: Enzyme-linked immunosorbent assay

Ig: Immunoglobulin

KLH: keyhole limpet hemocyanin

MAP: multiple antigen peptide

PBS: phosphate-buffered saline

SEA: *Staphylococcus aureus* enterotoxin A

TBST: TBS containing 0,1% Tween 20