

Presentation of Antigen in Immune Complexes Is Boosted by Soluble Bacterial Immunoglobulin Binding Proteins

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Summary

Using a snake toxin as a proteic antigen (Ag), two murine toxin-specific monoclonal antibodies (mAbs), splenocytes, and two murine Ag-specific T cell hybridomas, we showed that soluble protein A (SpA) from *Staphylococcus aureus* and protein G from *Streptococcus* subspecies, two Ig binding proteins (IBPs), not only abolish the capacity of the mAbs to decrease Ag presentation but also increase Ag presentation 20–100-fold. Five lines of evidence suggest that this phenomenon results from binding of an IBP–Ab–Ag complex to B cells possessing IBP receptors. First, we showed that SpA is likely to boost presentation of a free mAb, suggesting that the IBP-boosted presentation of an Ag in an immune complex results from the binding of IBP to the mAb. Second, FACS® analyses showed that an Ag–Ab complex is preferentially targeted by SpA to a subpopulation of splenocytes mainly composed of B cells. Third, SpA-dependent boosted presentation of an Ag–Ab complex is further enhanced when splenocytes are enriched in cells containing SpA receptors. Fourth, the boosting effect largely diminishes when splenocytes are depleted of cells containing SpA receptors. Fifth, the boosting effect occurs only when IBP simultaneously contains a Fab and an Fc binding site. Altogether, our data suggest that soluble IBPs can bridge immune complexes to APCs containing IBP receptors, raising the possibility that during an infection process by bacteria secreting these IBPs, Ag-specific T cells may activate IBP receptor-containing B cells by a mechanism of intermolecular help, thus leading to a nonspecific immune response.

Key words: Ag presentation • protein A • B cell superantigen

Previous studies have shown that an Ag-specific Ab can modulate presentation of an Ag to T cells (1–5). At least two mechanisms can be associated with these observations. First, some of the Fcγ receptors expressed on APCs can mediate the internalization of the immune complex and thus may enhance Ag presentation (6–8). Second, Ag-specific Abs can affect the processing pattern of an Ag (9, 10), causing an enhanced or suppressed presentation of various T cell determinants (11, 12). This raises the question of whether components that interact with Abs, such as soluble microbial Ig binding proteins (IBPs),¹ can also influence presentation of Ag–Ab complexes.

Microbial IBPs are produced by protozoa, viruses, and both gram-positive and gram-negative bacteria (13) and

play important physiological roles (14). In particular, protein A (SpA) from *Staphylococcus aureus* can modify opsonization, phagocytosis, complement consumption (15, 16), Ab-dependent cell-mediated cytotoxicity (17), and mitogenesis (18). Molecular properties of SpA are well documented. It is a monomeric protein that can bind to an Ig molecule, free or bound to its Ag (19–21). SpA can bind to the Fcγ part of the Ab (22) through any of its five Fcγ binding domains, called EDABC (23, 24). It can also bind to the Fab region of an Ab via an alternative recognition site (25–30) located in the V_H domain (31). This site is present in a large proportion of IgM, IgA, and IgG F(ab')₂ that are restricted to the V_HIII family in humans (32–34) and to the S107 and J606 families in mice (35, 36). Thus, SpA can interact with a large proportion of B cells and is therefore considered a B cell superantigen (37, 38). As SpA is naturally and efficiently secreted by *S. aureus* (39–41), we investigated its possible influence on presentation of an Ag–Ab complex.

¹Abbreviations used in this paper: CTLL, cytotoxic T cell line; FCC, fluorescein-5(6)-carboxamidocaproic; hIg, human immunoglobulin; IBP, immunoglobulin binding protein; SAPE, streptavidin-PE; SpA, staphylococcal protein A; spp., subspecies.

In this study, we examined, in a murine model, the influence of SpA on T cell presentation of Ag-Ab complexes, using a snake toxin as a proteic Ag, two toxin-specific mAbs, splenocytes, and two Ag-specific T cell hybridomas. We show that SpA not only abolishes the capacity of mAbs to diminish Ag presentation but also increases Ag presentation by 20–100-fold. In addition, we show that (i) SpA targets Ag-Ab complex to a subpopulation of splenocytes containing SpA receptors and mainly composed of B lymphocytes; (ii) APCs that possess SpA receptors are responsible for the boosting effect; (iii) the boosting effect occurs only when the IBP simultaneously possesses a Fab and an Fc binding site; (iv) in the absence of its Ag, an Ab also undergoes a SpA-specific boosted presentation. Altogether, our observations suggest that SpA boosts Ag presentation by bridging an immune complex to SpA receptors present at the surface of appropriate APCs. We also show that protein G from *Streptococcus* subspecies (ssp.) group C, another bacterial IBP, can also boost presentation of Ag-Ab complexes. Our observations raise the possibility that during an infection process by bacteria secreting these IBPs, Ag-specific T cells may activate IBP receptor-containing B cells by a mechanism of intermolecular help, thus leading to a nonspecific immune response. Several reports suggest that these observations may also be extended to humans.

Materials and Methods

Proteins and Reagents

Protein A from *S. aureus*, protein G from group C *Streptococcus* ssp., the IgG binding fragment BB, and protein G' were purchased from Sigma Chemical Co. ZZ was expressed and purified as previously described (49). Toxin α was purified from *Naja nigricollis* venom (Pasteur Institute, Paris, France). Purity of the toxin was assessed by both reverse phase HPLC and isoelectric focusing. Biorex 70 and Bio-Gel P2 were obtained from Bio-Rad Labs. *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) was purchased from Pharmacia, and *N*-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) was purchased from Pierce Chemical Co. *N*-hydroxysuccinimidyl-fluorescein-5(6)-carboxamidocaproate (NHS-FCC) was obtained from Sigma Chemical Co. The HPLC C18 column (25 cm \times 10 mm) was from Vydac. All solvents were obtained from Merck and used without further purification. mAb B220, Mac-2, anti-CD4, and anti-CD8 were from the American Type Culture Collection. Streptavidin-PE (SAPE) was purchased from Caltag Labs.

Chemical Modification of Proteins

Chemical Modification with NHS-FCC and NHS-biotin. SpA was dialyzed overnight at 4°C against PBS before its modification with NHS-FCC. 52 nmol of SpA was subsequently incubated for 1 h at room temperature with 10 \times excess NHS-FCC. The reaction mixture was filtered through a Sephadex G15 column (26 \times 1.5 cm) equilibrated in 50 mM phosphate buffer, pH 7.2. The protein that eluted in the void volume was used without further purification. 73.5 nmol of toxin α was modified with 735 nmol of NHS-FCC according to the same protocol. The procedure used for the biotinylation of protein G was similar to that described for NHS-FCC.

Covalent Coupling of Toxin α to mAb MST2

For the covalent coupling of toxin α to MST2, we used a modified procedure of the protocol previously described for coupling of toxin α to peroxidase (42).

Monothiolation of Toxin α . 200 μ l of SPDP (17 μ M) in absolute ethanol was added to 3.38 μ mol of toxin α in 1.8 ml of 0.1 M phosphate buffer, pH 7.5, containing 0.1 M NaCl. The mixture was stirred for 30 min at room temperature. The reaction mixture was filtered through a Bio-Gel P2 column (26 \times 1.5 cm) equilibrated in 10% acetic acid. The protein which eluted in the void volume was freeze dried. The modified protein was dissolved in 1 ml of 0.05 M ammonium acetate, pH 7.1, and applied onto a Bio-Rex 70 column (70 \times 2.5 cm) equilibrated in the same buffer. The toxin was eluted with a linear gradient of 0.05–0.4 M ammonium acetate, pH 7.1. Fractions containing mono-modified derivatives were roughly identified by analogy with the elution profile previously obtained with monoacetylated derivatives. The modified amino groups were unambiguously identified by determination of the affinities of each derivative for two toxin-specific mAbs. Each derivative was rechromatographed on a C18 10-mm Vydac column equilibrated in water containing 0.1% TFA. The derivatives were eluted by using acetonitrile as secondary solvent. Elution was over a linear gradient of 0–50% acetonitrile. Each derivative that was freeze dried had incorporated a 2-pyridyl-dithiopropionate moiety on a single lysine.

Incorporation of Maleimido Groups into mAb MST2. 5.5 μ l of dimethylformamide containing 83 nmol of SMCC was added to 8.3 nmol of MST2 dissolved in 312 μ l of 0.1 M phosphate buffer, pH 7, containing 0.1 M NaCl. The mixture was left for 1 h with stirring at room temperature. The reaction mixture was then filtered through a Sephadex G15 column (20 \times 0.5 cm) equilibrated in 0.1 M phosphate buffer, pH 6, containing 0.1 M NaCl.

Coupling Procedure. The coupling reaction was performed in three steps. (i) The extra disulfide bond at Lys 27 was reduced. 8.3 nmol of the derivative was dissolved in 200 μ l of 0.1 M acetate buffer, pH 4.5, containing 0.1 M NaCl and 25 mM dithiothreitol. The mixture was left under stirring at room temperature for 20 min, and the solution was filtered through a Sephadex G15 column (20 \times 0.5 cm) equilibrated in 0.1 M phosphate buffer, pH 6.1, containing 0.1 M NaCl. The monothiolated toxin eluted in the void volume. (ii) (27-N ϵ mono thio-propionyl-lysine)-toxin α (8.3 nmol) was reacted with maleimido MST2 (8.3 nmol) overnight at 4°C. Uncoupled toxin α was removed by affinity chromatography on a Sepharose-protein G column. The conjugate was then concentrated using a Centricon[®] 10 and stored at –20°C with 0.1% BSA.

ELISA

Microtiter ELISA plates were coated overnight with either 0.3% BSA or polyclonal human (h) IgMs (1 μ g/well) in 0.05 M phosphate buffer, pH 7.4. Plates were then saturated with 0.3% BSA in 0.1 M phosphate buffer, pH 7.4. Dilutions of biotinylated toxin α , M α 2-3, ZZ, and BB were performed in 0.1 M phosphate buffer, pH 7.4, containing 0.1% BSA. For the assessment of the binding of M α 2-3 to IgM-coated plates, the mAb (3 nM) was incubated overnight at 4°C in BSA-coated plates in the presence or absence of ZZ or BB (8 nM for each SpA derivative). The incubated solutions were then transferred into IgM-coated plates and left at room temperature for 4 h. The wells were then washed five times with 0.01 M phosphate buffer, pH 7.4, containing Tween 20, and a F(ab')₂ goat anti-mouse IgG peroxidase conjugate (Immunotech) was added and incubated for 30 min. After ex-

tensive washings, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) was added, coloration was developed for 30 min, and the resulting absorbance was measured at 414 nm. For the assessment of the binding of biotinylated toxin α to IgM-coated plates, M α 2-3 and biotinylated toxin α were incubated overnight at 4°C in BSA-coated plates in the presence or absence of ZZ or BB. The incubated solutions were then transferred into IgM-coated plates and left at room temperature for 4 h. The wells were washed, and a streptavidin peroxidase conjugate (Immunotech) was added and incubated for 30 min. ABTS was used as a substrate as described above.

T Cell-stimulating Assay

All experiments were performed using DCCM1 (Biological Industries) as a synthetic culture medium, in the absence of FCS. Fixed or serial dilutions of the different Ags were preincubated in microculture wells for one night at 4°C. 5×10^5 splenocytes per well were then added, along with 5×10^4 of either T1B2 or T1C9 hybridoma. Cells were cultured for 24 h at 37°C in a humidified 7% CO₂ atmosphere. For the assessment of the presenting capacity of SpA-specific cells, various amounts of splenocytes, splenocytes enriched with SpA-specific cells, or splenocytes depleted of SpA-specific cells were added to the wells in the presence of 5×10^4 T1B2.

The presence of IL-2 in culture supernatants from T cell hybridoma and bulk culture was evaluated by determining the proliferation of an IL-2-dependent cytotoxic T cell line (CTLL) using methyl [³H]TdR (5 Ci/mmol; CEA). The data are expressed in cpm.

Flow Cytometric Analysis

Binding to Splenocytes. Biotinylated toxin α (0.7 μ M) was incubated for one night at 4°C in the presence or absence of SpA and M α 1 (0.6 and 0.1 μ M, respectively). Splenocytes (5×10^5 cells) were then added and incubated for 30 min at 4°C in PBS/0.5% BSA. Cells were washed three times and incubated for 30 min at 4°C with SAPE (6 μ g/ml). After three washes, 5,000 viable cells were analyzed on a FACS® (Becton Dickinson). The binding of SpAF to splenocytes was assessed by incubating 5×10^5 cells for 30 min at 4°C with 50 μ l SpAF (0.6 μ M final) in the presence or absence of $10 \times$ excess unlabeled SpA. After three washes, 5,000 viable cells were analyzed on a FACS®. For the assessment of the binding of the two IBPs to splenocytes, biotinylated protein G (0.2 μ M final) was incubated for 30 min at 4°C with 5×10^5 cells in the presence or absence of SpAF. Splenocytes were subsequently washed three times and incubated for 30 min at 4°C with SAPE (6 μ g/ml). After three washes, 5,000 viable cells were analyzed on a FACS®.

Cell Sorting of Splenocytes. Cells were incubated for 30 min at 4°C in the presence of SpAF. After three washings, splenocytes were filtered through nylon and sorted at 2,000 cells/s under sterile conditions on an EPICS V flow cytometer (Coulter) using a 76- μ m nozzle and the 488-nm line of an argon laser set at 300 mW. PBS was used as a sheath fluid. The sorting criteria were normal forward-angle light scatter and bright fluorescein fluorescence (515–560 nm) taken on a log scale; $\sim 2\%$ of cells were positive.

Binding to Cell-sorted Splenocytes. Biotinylated toxin α (0.7 μ M) and SpA (0.6 μ M) were incubated for one night at 4°C in the presence or absence of M α 1 (0.1 μ M). 10^5 splenocytes, SpA unbound splenocytes, or SpA-bound cell-enriched splenocytes were then added and incubated for 30 min at 4°C in PBS/0.5% BSA. Cells were washed three times, and binding to cells was analyzed as described above. To determine the type of cells bound by SpAF,

SpA-bound cell-enriched splenocytes were incubated for 30 min at 4°C with mAb-B220, Mac-2, anti-CD4, and anti-CD8, respectively (0.1 μ M final for each). Cells were washed three times, and binding to cells was analyzed as described above.

Results

SpA Enhances Presentation of Ag-Ab Complex. We investigated the influence of SpA from *S. aureus* on presentation of an Ag-Ab complex to Ag-specific T cells. We used a snake toxin, toxin α (43), as an Ag, two toxin α -specific T cell hybridomas, called T1C9 and T1B2 (44), and two IgG2a toxin α -specific mAbs, called M α 1 (45) and M α 2-3 (46), whose epitopes are topographically unrelated to each other. Serial dilutions of toxin α were preincubated overnight at 4°C in the presence of fixed quantities of a toxin-specific mAb (M α 1 or M α 2-3) and SpA before addition of the appropriate T cells and splenocytes from BALB/c mice. Secretion of IL-2 was determined 24 h later. As shown in Fig. 1, the capacity of toxin α to stimulate T1B2 and T1C9 in the presence of splenocytes decreased when the Ag was incubated with either M α 1 (Fig. 1, A and B) or M α 2-3 (Fig. 1 C). However, the suppressing effect caused by the two toxin-specific mAbs not only vanished in the presence of soluble SpA, but the efficacy of Ag presentation dramatically increased. Fig. 1 shows that 20–100-fold lower amounts of toxin α sufficed to cause secretion of the same amounts of IL-2 when the Ag was incubated with both toxin-specific mAbs and SpA. Therefore, the presence of an Ig-binding protein can specifically enhance presentation of an Ag complexed to an Ag-specific Ab.

In these experiments, we used relatively high proportions of SpA and mAbs (0.2 μ M). Nevertheless, when their concentrations decreased to 4 nM and 2 nM, respectively, being thus probably closer to physiological conditions, a potent boosting effect remained observable (Fig. 1 D). It is notable that, in these experiments, the initial suppressing effect of the mAb apparently disappeared (Fig. 1 D). This is no surprise, as the ratio of concentrations of mAb (2 nM) to antigen (0.1 μ M) was low and, as a result, the presentation associated with the free toxin dominated and overshadowed the inhibiting effect of the mAb. The same phenomenon was observed in all experiments in which the toxin concentration was higher than that of the antibody (see below).

This is not the first time that an Ag presentation has been shown to be affected by mAbs. In particular, presentation of tetanus toxin was shown to increase or decrease in the presence of some specific mAbs (11, 12). In our study, we observed only a decrease in presentation by both toxin-specific mAbs, although they bound to topographically unrelated epitopes (45, 46). This result might be associated with the small size (6.8 kD) of the antigen, whose mAbs cover a large proportion of the toxin surface and are thus likely to interfere with presentation of the toxin T cell epitopes, located between residues 24–41 and 32–49 (44).

It was previously shown that when SpA is expressed at the surface of *Staphylococcus* or coupled to Sepharose, it can exert a mitogenic stimulating effect (18, 47). To rule out

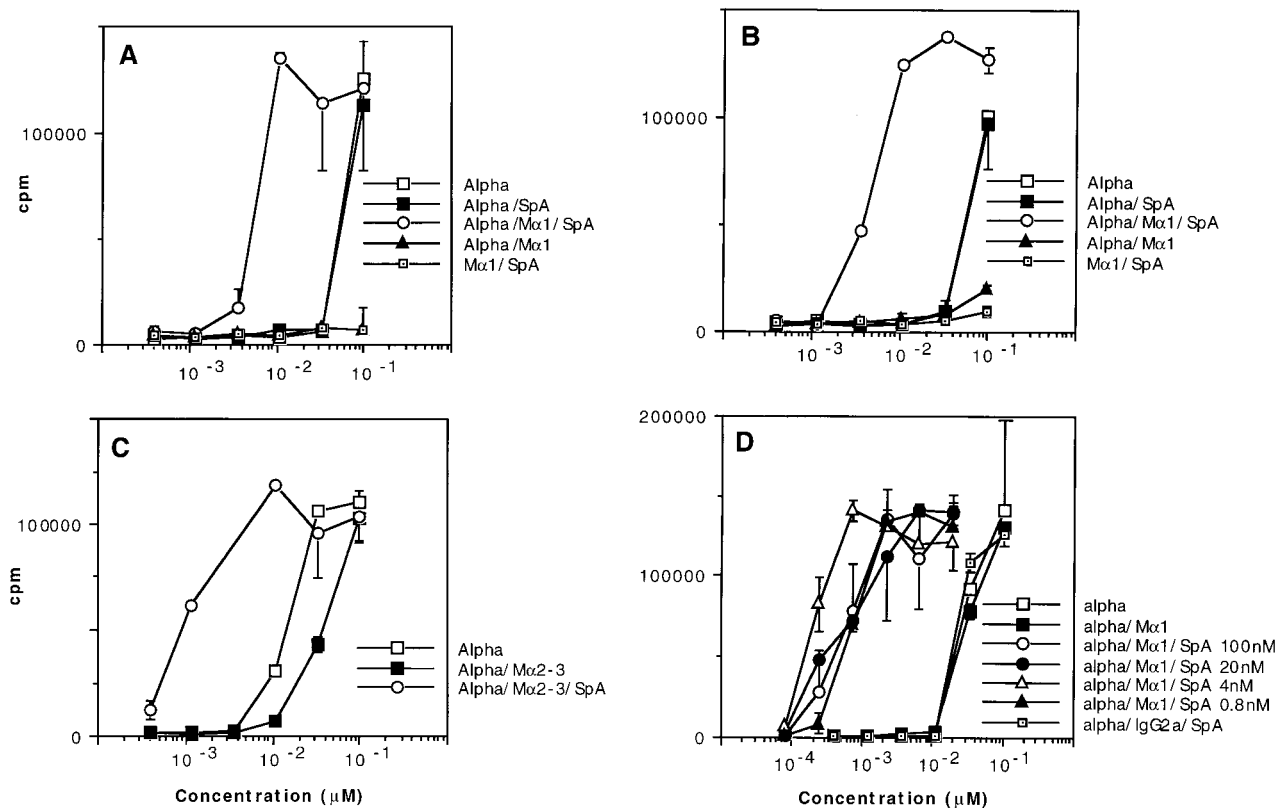


Figure 1. T cell presentation of toxin α in the presence of soluble SpA. Toxin α (Alpha) was serially diluted and incubated overnight at 4°C in the presence or absence of fixed concentrations of SpA and either mAb M α 1 (0.2 μ M final) or M α 2-3 (0.1 μ M final). 5×10^5 splenocytes from BALB/c mice were then added to each well in the presence of 5×10^4 of either T1B2 (A) or T1C9 (B and C). Cells were cultured for 24 h at 37°C, and IL-2 secretion was subsequently determined by CTLL assay. The experiments shown in A were repeated in the presence of variable concentrations of SpA and a fixed concentration of M α 1 (2 nM final; D). In this experiment, a control was performed using an unrelated IgG2a and SpA (0.1 μ M final for each).

the possibility that the SpA-specific boosting phenomenon described above was related to such a nonspecific property, various controls were performed. First, in the absence of any mAbs, M α 1, or M α 2-3, the toxin α stimulation efficacy was unaffected by SpA (Fig. 1, A and B). Second, in the absence of toxin α , no IL-2 stimulation was triggered by soluble SpA and M α 1 (Fig. 1, A and B). Third, SpA did not modify Ag presentation when the Ab was an unrelated IgG2a (Fig. 1 D). Fourth, no stimulation occurred when splenocytes and T1B2 were incubated separately with SpA, toxin α , and M α 1 (data not shown). Therefore, besides the

necessary appropriate immune cells, three additional components were absolutely required for the boosting effect to be observable: the Ag, an Ag-specific Ab, and soluble SpA.

The SpA-boosted Presentation of Ag Remains MHC Restricted. The presentation of free toxin α to T1B2 and T1C9 is restricted to I-A^d and I-E^d, respectively (44). We investigated whether or not this MHC restriction was still associated with the SpA-boosted presentation of the toxin using splenocytes from D2GD mice, which express I-A^d and I-E^b molecules. We observed that the capacity of toxin α to stimulate T1B2 was boosted when the toxin was added

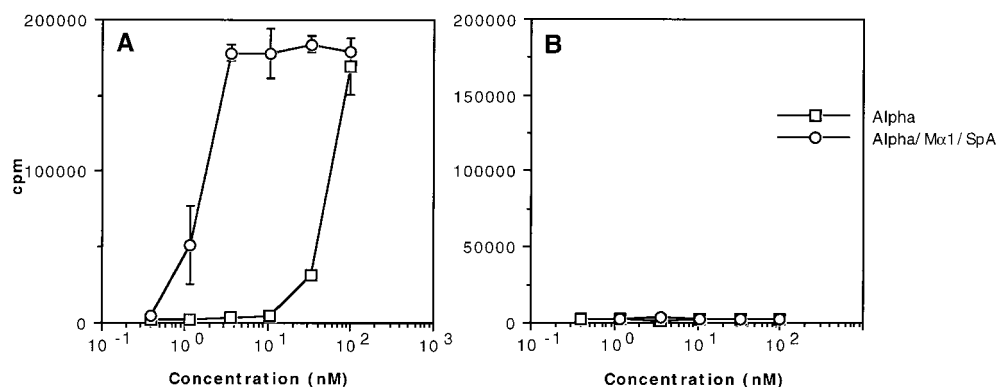


Figure 2. T cell presentation of toxin α in the presence of toxin-specific antibody and SpA is MHC restricted. Toxin α was serially diluted and incubated overnight at 4°C in the presence or absence of fixed concentrations of SpA and M α 1 (0.1 μ M final for each protein). 5×10^5 of splenocytes from D2GD mice were then added to each well in the presence of 5×10^4 of either T1B2 (A) or T1C9 (B). T cell stimulation was assessed as in the Fig. 1 legend.

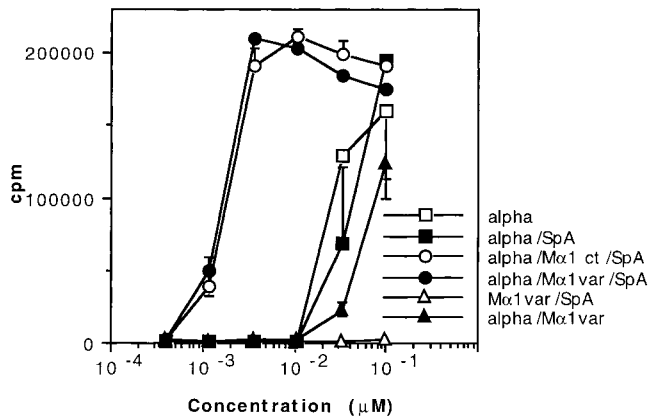


Figure 3. T cell presentation of toxin α in the presence of fixed or decreasing concentrations of $M\alpha 1$. Toxin α was serially diluted and incubated overnight at 4°C in the presence or absence of fixed concentrations of SpA ($0.3 \mu\text{M}$ final) and either serial dilutions of $M\alpha 1$ or a fixed amount of mAb ($0.2 \mu\text{M}$ final). 5×10^5 splenocytes from BALB/c mice were then added to each well in the presence of 5×10^4 T1B2. T cell stimulation was then assessed as previously described.

concomitantly with both the mAb and SpA (Fig. 2 A), whereas under the same experimental conditions, T1C9 was not stimulated (Fig. 2 B). Therefore, the SpA-dependent boosting presentation of an Ag complexed to an Ag-specific Ab remains MHC restricted and is not related to the MHC-independent genetic background of BALB/c mice.

Ag-Ab Complexes Are Targeted to APC Possessing SpA Receptors. What are the mechanisms that may account for an SpA-dependent boosting presentation of an Ag complexed to an Ag-specific Ab? As already mentioned, SpA possesses multiple Ig binding sites (23, 24) and therefore could bind simultaneously to several immune complexes, thus generating an artificial concentration effect. In other words, incubation of stoichiometric concentrations of Ab and SpA could favor formation of heterogeneous multimolecular complexes, and those possessing several toxin molecules could predominantly generate the boosting effect. To examine

such a possibility, we incubated overnight a constant concentration of SpA with varying dilutions of toxin α and either a fixed concentration of $M\alpha 1$ or varying concentrations of $M\alpha 1$ so that the ratios of SpA/ $M\alpha 1$ were equal to 1.5, 4.5, 13.5, 40.5, 121.5, or 364.5 for the first to the sixth dilution, respectively. Thus, in each toxin dilution, at most one molecule of the immune complex should have bound to one SpA molecule. As shown in Fig. 3, T cell presentation evolved similarly with toxin dilution, irrespective of whether the concentrations of $M\alpha 1$ were fixed or variable. Therefore, the observed boosted T cell stimulation seems to be unrelated to the number of Ab-Ag molecules associated to an SpA molecule.

Another explanation that may account for the SpA-enhanced presentation of an Ag is that SpA targets Ag-Ab complexes to APCs that possess SpA receptors on their surfaces. To investigate this possibility, we performed a series of FACS[®] analyses. At first, we examined the direct binding of SpA to splenocytes using SpA labeled with FCC (SpAF). As shown in Fig. 4, E-G, labeled SpA binds specifically to a subpopulation of splenocyte cells. We then investigated the binding of the Ag on splenocytes using SpA, a toxin-specific mAb ($M\alpha 1$), and the biotinylated toxin α , which was detected by SAPE. When the biotinylated toxin α was added alone, $<0.5\%$ of splenocytes were labeled (Fig. 4 A). The same result was obtained when SpA (Fig. 4 B) or $M\alpha 1$ (Fig. 4 C) was added separately. In sharp contrast, when the biotinylated toxin was concomitantly incubated with $M\alpha 1$ and SpA, the proportion of labeled cells increased to nearly 3% (Fig. 4 D). These findings support the view that splenocytes possess SpA-specific cells and that an Ag-Ab complex binds to this subpopulation.

To further validate this conclusion, we sorted SpAF-labeled splenocytes (2% of the whole population) and investigated the binding of the biotinylated Ag to this population enriched in SpA-specific cells. 32% of the positively sorted splenocytes were effectively labeled with SpAF (Fig. 5 E, bottom right). In the absence of both SpA and $M\alpha 1$, quite a small proportion of these cells were labeled with the

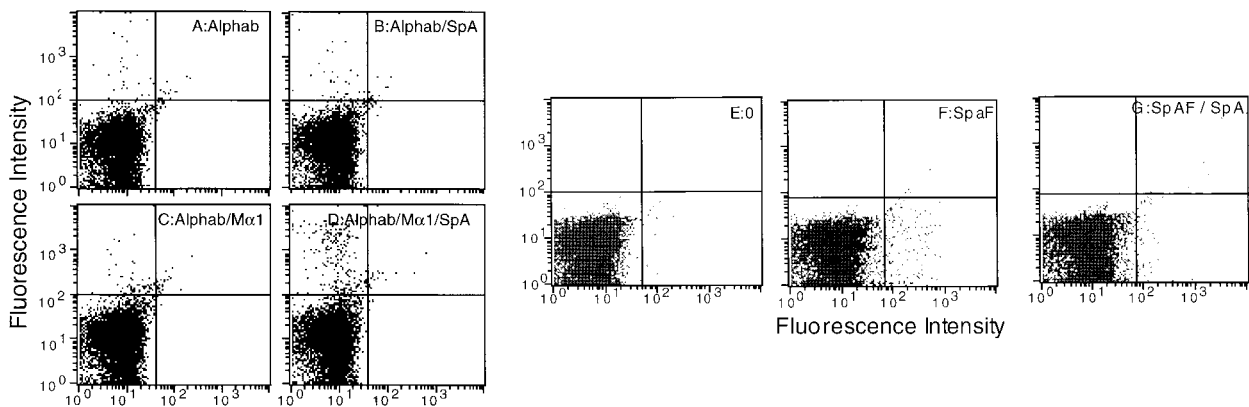


Figure 4. Binding of the immune complex and SpA on splenocytes. Biotinylated toxin α (Alphab) was incubated overnight at 4°C (A), in the presence of a fixed concentration of SpA (B), $M\alpha 1$ (C), or both (D). Splenocytes (5×10^5 cells) were added and incubated for 30 min at 4°C . Binding of biotinylated toxin α to cells was assessed using SAPE. FACS[®] analyses E-G show splenocytes (5×10^5 cells) incubated for 30 min at 4°C without SpAF (E), in the presence of SpAF alone (F), or SpAF diluted with a $10\times$ excess unlabeled SpA (G).

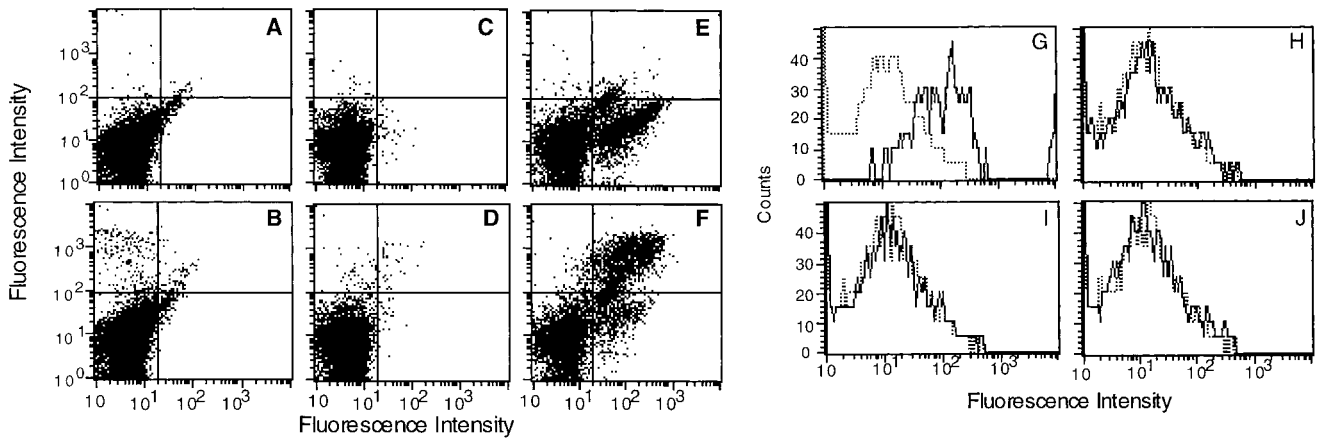


Figure 5. Binding of toxin α on cell-sorted splenocytes. Biotinylated toxin α and SpA were incubated overnight at 4°C with (B, D, and F) or without (A, C, and E) M α 1. 10^5 unsorted splenocytes (A and B), splenocytes depleted of cells labeled with SpA-FCC (C and D), or splenocytes enriched in cells labeled with SpA-FCC (E and F) were added and incubated for 30 min at 4°C. Binding of biotinylated toxin α to cells was assessed using SAPE. Analyses G–J show characterization of splenocytes enriched in cells labeled with SpAF, using biotinylated mAbs B220, Mac-2, GK1.2, and H35 (G, H, I, and J, respectively).

biotinylated toxin (Fig. 5 E, top right). In contrast, when both M α 1 and SpA were present, nearly 60% of the SpAF-labeled cells were labeled with the biotinylated Ag (Fig. 5 F, top right). However, a proportion of SpAF-labeled splenocytes were not labeled with the biotinylated toxin, even when both the mAb and SpA were present in the medium (Fig. 5 F, bottom right). No definite explanation presently accounts for this observation. Control experiments were carried out with the population of negatively sorted cells, which contained only 0.4% of SpAF-labeled cells (Fig. 5 C, bottom right). Only 0.6% of the depleted splenocytes were labeled with the biotinylated Ag in the presence of the Ab and SpA (Fig. 5 D, top left and top right), and this value did not increase to more than 2.5% when the splenocytes were unsorted (Fig. 5 B, top left). Altogether, these experiments provide direct evidence that in the presence of SpA, an Ag–Ab complex is preferentially targeted to a particular subpopulation of splenocytes that possess SpA receptors.

To identify the cell types that predominantly populated splenocytes enriched in sorted SpAF-labeled cells, we used four biotinylated mAbs (mAb B220, mAb Mac 2, mAb GK1.2, and mAb H35) and SAPE. As shown in Fig. 5 G, a shift in fluorescence intensity was observed with the B cell marker, whereas no shift was observed using macrophage, CD4, and CD8 cell surface markers (Fig. 5, H–J). Therefore, SpAF-bound cells are mainly composed of B cells.

The Boosted Presentation Requires APCs Possessing SpA Receptor. We investigated the ability of both unsorted splenocytes (S) and splenocytes enriched with (ES) or depleted of (DS) SpAF-bound cells to stimulate T1B2 in the presence of toxin α alone or in combination with both SpA and M α 1. As shown in Fig. 6 A, 10^4 splenocytes enriched with SpA receptor–possessing cells were ~ 10 -fold more efficient than 10^4 unsorted splenocytes at boosting toxin α presentation in the presence of M α 1 and SpA. In contrast, 10^5 unsorted splenocytes were ~ 10 -fold more efficient than 10^5 splenocytes depleted of SpA receptor–containing cells (Fig.

6 B). The observed differences in efficiency are not related to a modification of the processing efficiency of cells treated with SpAF during the sorting experiments, as control experiments showed that splenocytes enriched with SpA receptor–containing cells were equally as efficient as unsorted splenocytes at presenting toxin α alone (Fig. 6). Further demonstration that positively sorted cells are more efficient than unsorted cells, which in turn are more efficient than the negatively sorted splenocytes, is also shown in Fig. 6 C, where stimulation of T1B2 by the Ab–Ag complex in the presence of SpA is shown as a function of the number of cells. Therefore, the subpopulation of splenocytes possessing SpA receptors is mainly responsible for the capacity of SpA to boost the T cell stimulation of Ag–Ab complex.

SpA Receptors May Be Surface Immunoglobulins. The above data indicated that the Ag–Ab complexes are targeted to SpA receptors present at the surface of APCs containing predominantly B cells. In other words, the boosting effect can be interpreted as resulting from the formation of complexes that consist of a SpA receptor bound to SpA, which in turn is bound to Ab–Ag complex. To investigate the possibility that the SpA receptors may be surface Igs, we proceeded as follows. First, we selected two SpA derivatives which differ in their recognition specificity toward Igs. One of them is composed of two B domains (BB), and, like SpA, recognizes the Fab region of IgMs and IgGs as well as the Fc γ part of IgG (48). The other derivative is ZZ (Z is a mutant of domain B [49]) and binds to the Fc γ part of IgG but very weakly to the Fab region (50). Second, we examined the binding of either a mouse mAb or biotinylated toxin α to hIgM-coated plates. As shown in Fig. 7 A (left), the mouse IgG2a mAb M α 2-3 can bind to coated hIgMs in the presence of BB but not in the presence of ZZ. Furthermore, the presence of M α 2-3 and BB is a prerequisite for the binding of biotinylated toxin α to coated hIgMs (Fig. 7 A, right). Therefore, formation of a ternary complex, IgM–SpA derivative–IgG, or a quaternary com-

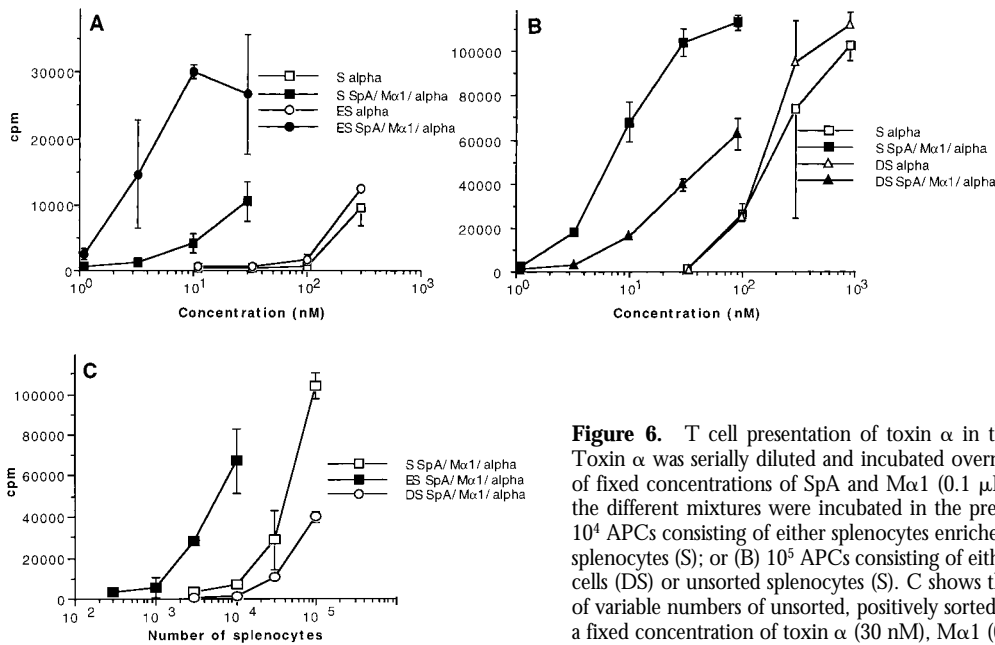


Figure 6. T cell presentation of toxin α in the presence of cell-sorted splenocytes. Toxin α was serially diluted and incubated overnight at 4°C in the presence or absence of fixed concentrations of SpA and M α 1 (0.1 μ M final concentration for both). Then, the different mixtures were incubated in the presence of T1B2 (5×10^4 cells) and (A) 10^4 APCs consisting of either splenocytes enriched in SpA specific cells (ES) or unsorted splenocytes (S); or (B) 10^5 APCs consisting of either splenocytes depleted of SpA-specific cells (DS) or unsorted splenocytes (S). C shows the stimulation of T1B2 in the presence of variable numbers of unsorted, positively sorted, and negatively sorted splenocytes, and a fixed concentration of toxin α (30 nM), M α 1 (0.1 μ M), and SpA (0.1 μ M).

plex, IgM–SpA derivative–IgG–Ag, is possible, provided that the derivative can bind to both IgG and IgM. In addition, the V_H domain of M α 2-3 is coded by the V_H germline gene J558 (51), a characteristic that precludes the Fab of this IgG to bind SpA and BB (35, 36). As a result, this IgG can be recognized only through its Fc γ . Therefore, the complex observed in the ELISA consists of an IgM bound by its Fab to BB, which in turn binds to the Fc γ region of M α 2-3.

Third, we investigated the effect of the two SpA derivatives on the labeling of splenocytes from BALB/c mice by a mouse IgG–FITC. In these unpublished experiments, we observed that 10.8% of the splenocytes were bound by the mouse IgG2a–FITC, a proportion which increased to 23.8% in the presence of BB and decreased to 6.4% in the presence of ZZ. Fourth, we investigated the capacity of BB and ZZ to boost the presentation of the M α 2-3–toxin complex using T1C9 and splenocytes. As shown in Fig. 7 B, a boosting effect is observed when the Ab–Ag complex

is incubated in the presence of BB but not in the presence of ZZ. Thus, the boosting effect requires that the SpA derivative possesses both a binding site to the Fc γ region of M α 2-3 and a binding site to the Fab of Igs, strongly suggesting that the BB–M α 2-3–Ag complex is targeted to the Fab region of surface Igs of APCs.

The Presentation of an Immune Complex Is Also Boosted by Protein G. We investigated whether a bacterial IBP other than SpA could also trigger a boosting effect. Several of the above experiments were therefore repeated using protein G from *Streptococcus* ssp. group C (52), which contains both a Fab binding site and an Fc γ binding site (53, 54). As shown in Fig. 8 A, protein G boosted presentation of toxin α to T1B2 only when the toxin, M α 2-3, and protein G were concomitantly added to appropriate immune cells. In contrast, the boosting effect was not observed with protein G', a protein G derivative that binds exclusively to the Fc γ of Igs (55). Therefore, these data suggest that the boosted pre-

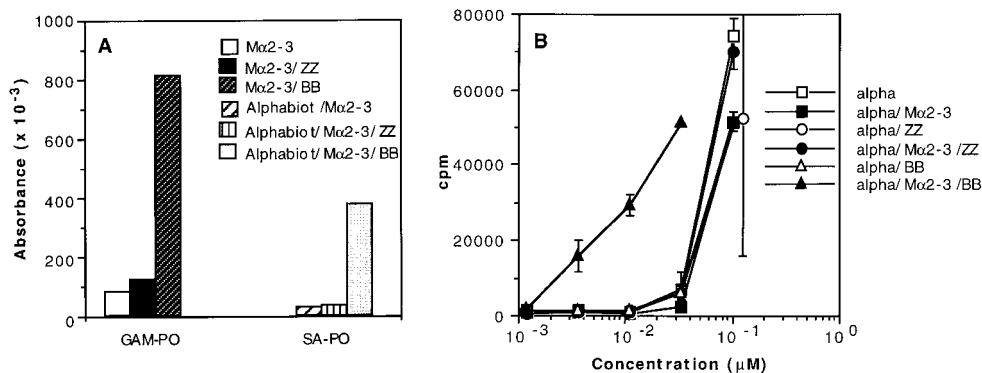


Figure 7. Effect of two SpA derivatives on the binding to coated hIgMs and on the T cell presentation of toxin α . (A) M α 2-3 was incubated overnight in the presence or absence of fixed concentrations of toxin α biotinylated at the NH₂ terminus (Alpha biot) and either ZZ or BB in BSA-coated microwell plates. The solutions were transferred in hIgM-coated plates. Binding of M α 2-3 to the wells was determined using a goat anti-mouse IgG peroxidase conjugate (GAM-PO), whereas binding of biotinylated toxin α was determined using a streptavidin peroxidase conjugate (SA-PO). (B) Toxin α (alpha) was serially diluted and incubated overnight at 4°C in the presence or absence of mAb M α 2-3 (25 nM final) and either ZZ or BB (0.1 μ M final for each derivative). 5×10^5 splenocytes from BALB/c mice were then added to each well in the presence of 5×10^4 T1B2. T cell stimulation was assessed as previously described.

termined using a streptavidin peroxidase conjugate (SA-PO). (B) Toxin α (alpha) was serially diluted and incubated overnight at 4°C in the presence or absence of mAb M α 2-3 (25 nM final) and either ZZ or BB (0.1 μ M final for each derivative). 5×10^5 splenocytes from BALB/c mice were then added to each well in the presence of 5×10^4 T1B2. T cell stimulation was assessed as previously described.

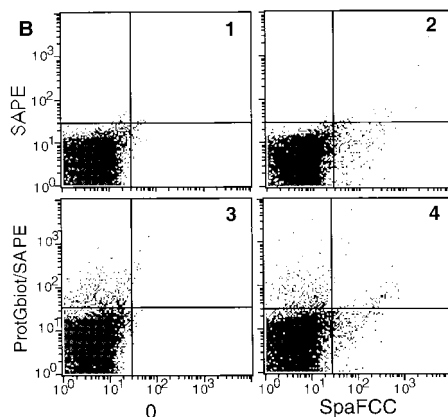
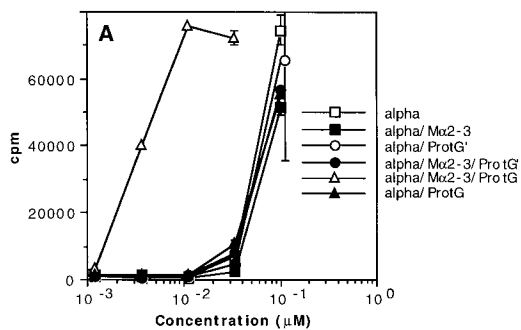


Figure 8. T cell presentation of toxin α in the presence of protein G from *Streptococcus* spp. (A) Toxin α (alpha) was serially diluted and incubated overnight at 4°C in the absence or presence of fixed concentrations of mAb M α 2-3 (25 nM final) and protein G or protein G' (0.1 μ M final concentration for each derivative). 5×10^5 splenocytes from BALB/c mice were then added to each well in the presence of 5×10^4 T1B2. Cells were cultured for 24 h at 37°C, and IL-2 secretion was subsequently determined by CTLL assay. (B) Splenocytes (5×10^5

cells) were incubated in the absence of IBP (B1) or presence of biotinylated protein G (protGbiot; B2), SpA-FCC (B3), or both (B4) for 30 min at 4°C. Cells were all incubated with SAPE and analyzed by flow cytometry.

presentation of an Ab-Ag complex results from a targeting by protein G to the Fab region of surface Igs of APCs. This raises the question of whether SpA and protein G recognize the same APC subpopulation. To approach this question, we performed FACS® analyses using both biotinylated protein G and SpAF. As shown in Fig. 8, protein G (B3, top left) and SpAF (B2, bottom right) bind to approximately the same proportion of the whole splenocyte population. Furthermore, the data presented in Fig. 8 (B4) suggest that SpA and protein G may recognize different splenocyte subpopulations. However, as we observed a slight increase in the upper right area of B4, we cannot preclude that a proportion of cells recognized by SpA and protein G are the same.

SpA May Also Boost T Cell Presentation of a Free Ab. The view that emerges from the above data is that presentation of an Ag is boosted in the presence of an Ag-specific Ab as a result of the targeting of the complex by an IBP to APCs that contain IBP receptors. According to this scenario, both the mAb and SpA should also undergo a boosted presentation. This was demonstrated earlier to be the case for SpA (56). To provide evidence that it is also the case for the mAb, one would need mAb-specific T cells; however, they were unavailable in our laboratory. To overcome this difficulty, we used the toxin α -specific T1B2 to tentatively monitor the presentation of an mAb that was selected for its inability to recognize toxin α (57). This mAb, an IgG1 called MST2, was covalently linked to toxin α , which became, therefore, a label to probe the presentation of MST2. As shown in Fig. 9, the MST2-toxin α couple was efficiently presented to T1B2, and its presentation was clearly boosted by SpA. As a control, no such boosting effect was observed when the free toxin α was incubated with both the uncoupled MST2 and SpA (not shown). Therefore, only the toxin that was covalently linked to the Ab underwent a boosted presentation. This effect most probably reflects a boosting effect of the Ab itself. We conclude, therefore, that the boosting presentation of an Ab-Ag complex, as previously perceived via the stimulation of the Ag, reflects an increased presentation of both the Ag and Ag-specific Ab, a finding in agreement with the proposed scenario

that an immune complex may be targeted by SpA to APC containing SpA receptors.

Discussion

Using a snake toxin as an Ag and two toxin-specific mAbs (M α 1 and M α 2-3), we showed that two soluble bacterial IBPs, SpA and protein G, dramatically increase the Ag-specific T cell stimulation of Ag-Ab complex. These findings are in sharp contrast to the observation that, in the absence of IBP, either of the Ag-specific mAbs inhibits presentation of the Ag.

A number of observations cast some light on the origin of this IBP-dependent boosting phenomenon. Thus, for the boosted presentation of the Ag to be seen, the Ag, an Ag-specific Ab, and an IBP, SpA or protein G, are absolutely required. As SpA is likely to boost presentation of a free Ab, we propose that the SpA-dependent boosted presentation of an immune complex, as perceived through presentation of its Ag, results from the binding of SpA to the Ab of the immune complex. Three lines of evidence indicate that the Ag-boosted presentation may result from binding of a ternary complex SpA-Ab-Ag to B cells containing SpA receptors. First, FACS® analyses demonstrated

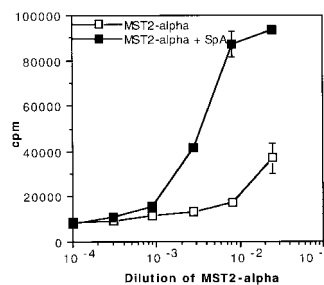


Figure 9. Boosted presentation of MST2, an mAb which does not recognize toxin α as an antigen. In the absence of MST2-specific T cell hybridoma, we took advantage of the availability of T1B2 and coupled MST2 covalently to toxin α , which, therefore, was a labeling probe to monitor the presentation of the mAb. The covalent MST2-toxin α complex (called

MST2-alpha) was serially diluted and incubated overnight at 4°C in the presence or absence of 0.2 μ M SpA. 5×10^5 splenocytes from BALB/c mice were added to each well in the presence of 5×10^4 T1B2. Cells were cultured for 24 h at 37°C, and IL-2 secretion was subsequently determined by CTLL assay.

that an Ag–Ab complex is preferentially targeted by SpA to a subpopulation of splenocytes possessing SpA-specific receptors and mainly composed of B cells. Second, the boosted presentation further increased when splenocytes were enriched in cells containing SpA-specific receptors. Third, the boosting effect largely decreased when APCs were depleted of cells containing SpA-specific receptors. Altogether, these observations suggest that the two soluble IBPs can bridge an immune complex to IBP receptors present at the surface of a particular subpopulation of APCs, thus enhancing endocytosis and presentation of the Ag.

For SpA to bridge an immune complex to SpA receptors, appropriate binding sites must exist on the Ag–Ab complex. It is known that SpA can interact with the Fc γ region of mouse IgGs without impairing the binding of the Ags to their paratopes (19). In addition, SpA can bind to an alternative site located within the Fab domain of some mouse Igs (25, 29) that is coded by V_H families S107 or J606 (35, 36). The V_H domain of M α 2-3, one of the two toxin-specific mAbs that triggered the boosting effect, is coded by the V_H germline gene J558 (51), whose gene product is, therefore, not recognized by SpA (35, 36). Thus, the Fc γ region of M α 2-3 and perhaps of M α 1, whose amino acid sequence is still unknown, are likely to be responsible for the interaction of the immune complex with SpA.

For SpA to bridge the Ab–Ag complexes to APCs, these cells must also possess appropriate SpA receptors. Four lines of evidence suggest that these receptors are surface Igs and most probably IgMs. First, we observed that the quaternary complex IgM–SpA (or BB)–M α 2-3–Ag can occur in vitro. Second, binding of a soluble IgG to splenocytes is increased only in the presence of an IBP derivative that possesses the capacity of binding concomitantly to Fab and Fc γ moieties. Third, for the boosting effect to be observed, an IBP with the same characteristics is also strictly required. Fourth, SpA preferentially targets Ab–Ag complex to a subpopulation of B cells. Because the surface IgMs offer an alternative binding site on the Fab domain, we suggest that they constitute the most plausible SpA receptors. Other possible SpA receptors are the Igs bound in vivo to FcRs present at the surfaces of macrophages (58). Although we detected no macrophages in the subpopulation of splenocytes enriched in cells containing SpA receptor, we cannot rule out their participation in the boosting effect. Therefore, our findings are compatible with the view that SpA may link the Fc γ part of the Ab moiety of an immune complex to cell surface, SpA-specific receptor structures such as IgMs present at the surface of a subpopulation of murine splenocytes.

Protein G from *Streptococcus* spp. also efficiently boosts the presentation of Ag in immune complexes. Like SpA, protein G binds to the Fc region of IgGs in the C γ 2–C γ 3 interface (53, 54), their respective epitopes partially overlapping (59). Unlike SpA, however, protein G binds to Fab regions of IgG via the CH1 domain (60, 61) and not to IgMs (52). Therefore, if soluble protein G acts, like SpA, as an enhancer of immune complex presentation, its bridging

capacity is likely to be different. Presumably, protein G predominantly focuses immune complexes to APCs carrying IgGs.

Several lines of evidence, mostly taken from the literature, indicate that SpA may react similarly with both human and mouse Igs. First, hIgs bind at two independent sites on SpA (14, 26–28, 30). Second, mouse Igs S107 and J606 are related to the human V_H genes of family 3 (62), and several works have shown that hIgG F(ab')₂ and hIgM reacting with SpA derive from the V_HIII family but do not have markers for other families (32–34). More precisely, 16/26 potentially functional germline V_HIII genes encode SpA reactivity (63, 64). Third, SpA–Sepharose or SpA-containing *S. aureus* can stimulate B cells expressing V_H gene segments encoded by a set of genes belonging to the V_HIII family (47). Fourth, as reported above, the quaternary complex hIgM–SpA–mAb–toxin is sufficiently stable to be detected in ELISA assays. Plausibly, therefore, soluble SpA could bridge the Fc region of soluble hIgs to cell surface hIgs coded by one or more of the 16 germline V_HIII genes encoding SpA reactivity. Remarkably, up to 54% of human B cells are capable of binding SpA (64), whereas V_H families J606 and S107 are expressed in only 10–20% of adult B lymphocytes of BALB/c mice (65–68) and are likely to bind SpA. Therefore, it is not impossible that the binding of SpA with hIgs could cause a boosting effect in humans similar to the one that we described here for mice.

During an humoral immune response, an Ag is captured by dendritic cells, macrophages, and Ag-specific B cells, processed, and presented to Ag-specific T helper cells. This cascade of events leads to Ab secretion. The secreted Ag-specific Abs bind to the Ag, forming an heterogeneous population of immune complexes. It has been proposed that these complexes can cause a negative feedback on the specific antibody response, as a result of the diversion of Ags from their specific B cells, toward cells possessing Ig receptors, i.e., Fc γ R-expressing cells and RF-producing B cells (69). In this scenario, the Ag-specific helper T cells may activate B cells producing RF by intermolecular help. The data presented in this paper suggest another mechanism that might affect the immune response during an infection process by IBP-secreting bacteria. As a result of the presence of a soluble IBP, SpA for example, the Ab–Ag complexes may be targeted to B cell IgMs that can bind SpA. The SpA-binding B cells may thus present T cell epitopes from the targeted immune complexes and stimulate the corresponding T cells and may be activated by an intermolecular help mechanism. Although their specific Ags may not necessarily be present in the medium, the targeted B cells may nevertheless secrete their natural antibodies. The specificities of the produced antibodies cannot be identified a priori. However, they are likely to be large because the V_H-reactive B cells are not bound by SpA via the traditional antigen binding site. It is tempting to speculate that this predicted diverting mechanism by an IBP may help the bacteria to evade the immune response.

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