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Human Secretory IgM Antibodies Activate Human Complement and Offer Protection at Mucosal Surface

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Abstract

IgM molecules circulate in serum as large polymers, mainly pentamers, which can be transported by the poly-Ig receptor (pIgR) across epithelial cells to mucosal surfaces and released as secretory IgM (SIgM). The mucosal SIgM molecules have non-covalently attached secretory component (SC), which is the extracellular part of pIgR which is cleaved from the epithelial cell membrane. Serum IgM antibodies do not contain SC and have previously been shown to make a conformational change from 'a star' to a 'staple' conformation upon reaction with antigens on a cell surface, enabling them to activate complement. However, it is not clear whether SIgM similarly can induce complement activation. To clarify this issue, we constructed recombinant chimeric (mouse/human) IgM antibodies against hapten 5-iodo-4-hydroxy-3-nitro-phenacetyl (NIP) and in addition studied polyclonal IgM formed after immunization with a meningococcal group B vaccine. The monoclonal and polyclonal IgM molecules were purified by affinity chromatography on a column containing human SC in order to isolate joining-chain (J-chain) containing IgM, followed by addition of excess amounts of soluble SC to create SIgM (IgM J+ SC+). These SIgM preparations were tested for complement activation ability and shown to be nearly as active as the parental IgM J+ molecules. Thus, SIgM may offer protection against pathogens at mucosal surface by complement-mediated cell lysis or by phagocytosis mediated by complement receptors present on effector cells on mucosa.

Introduction

IgM is the dominating immunoglobulin class produced during a primary immune response and also constitutes a major fraction of the so-called natural antibodies, which may have an important regulatory role in health and disease [1]. Furthermore, IgM and IgG are the only classes of immunoglobulins that efficiently induce complement activation through the classical pathway, and IgM is often considered to be most potent in this respect [2]. However, we have recently observed equal complement activation ability of chimeric human IgM and IgG with identical V-regions providing specificity for an epitope on an outer membrane protein (OMP) of meningococci [3].

IgM and IgA are actively secreted to mucous membranes by engaging the pIgR and thereby forming SIgM and SIgA [4], although IgG is also found to be present at mucosa, especially in the lungs, due to FcRn-mediated transport [5]. The amounts of SIgA are normally higher than those of SIgM. However, IgA deficiency is not uncommon, with an estimated rate of 2% (for review see [6]), and afflicted individuals are healthy and without clinical symptoms. In such situations, the lack of SIgA is compensated by increased amounts of SIgM [7]. It appears that J-chain, which is present in IgM and polymeric IgA, is a crucial part of the pIgR recognition site on both molecules (for review see [4]), and thus, both SIgA and SIgM contain J-chain. IgM molecules in serum exist as pentamers or hexamers which might or might not contain J-chain [4, 8], and it is still an open question whether IgM molecules can contain more than one J-chain [9]. Once IgM molecules containing J-chain have bound to pIgR on epithelial cells, the complexes are transcytosed through the epithelial cells from the basolateral to the apical side, where the pIgR is attacked by membrane proteases, leaving the extracellular part of pIgR, named SC, as an integral part of SIgM (IgM J+ SC+) [4].

Antibody-dependent complement-mediated lysis (ADCML) induced by IgM molecules is initiated by the binding of IgM to the surface of target cells, followed by a conformational change into a 'staple' form that allows C1 binding and activation. This notion is based on electron

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microscope observation [10], and the IgM used for the studies was derived from serum and thus did not contain SC. The presence of J-chain in pentameric human IgM profoundly inhibits its ability to activate guinea-pig complement, but not human complement [8]. Thus, pentameric human IgM containing J-chain can activate human complement, while the influence of SC added during the transcytosis to the mucous membrane to form SIgM is not known.

To clarify this issue, we studied recombinant, chimeric IgM anti-NIP as well as polyclonal human IgM antimeningococcal antibodies formed during primary immunization, and we separated the antibodies into a J+ and a J- fraction by SC-affinity chromatography. We found that the IgM J+ molecules were less active than the IgM J− molecules in activating human complement, while the addition of SC to the IgM J+ fraction to reconstitute SIgM only moderately reduced its human complement activation activity. Thus, SIgM can offer protection at the mucosal surface because complement factors have been detected at mucosal surface/secretions [11–13]. Similar reconstitution of SIgM by adding external SC has recently been published, but without analysing the complement activation capability of the molecules [14].

Materials and methods

Construction of chimeric IgM antibodies. Chimeric IgM anti-NIP-producing cells were established by stable transfection of NS0 mouse myeloma cells with mouse Vλ1-anti-NIP/human Cκ and mouse Vh-anti-NIP/Cμ as previously described [15].

Quantitation of chimeric IgM in cell cultures and human sera after vaccination. The concentration of IgM was quantitated by ELISA using microtiter coated with $NIP_{15}BSA$ (1 μg/ml), or OMV (4 μg/ml)prepared from meningococcal group B strain H44/76 as previously described [3, 16]. Standards in the assay were purified chimeric IgM anti-NIP and antimeningococcal PorA P1.16, respectively [3, 17]. After incubation for 2 h at 37 °C, the plates were washed with PBS/T using a microplate washer (ScanWasher 300, Scantron, Lierbyen, Norway). Then, the plates were incubated with a mixture of locally produced, affinity-purified, biotin-labelled sheep anti-human IgM, streptavidin and biotin-labelled alkaline phosphatase. After incubation for 2 h at 37 °C, the plates were again washed with PBS/T and developed with 100 μl 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer pH 9.8. The plates were incubated for 30–60 min at 37 °C, and the absorbance was recorded at 405 nm, using a microplate reader (Thermomax, Molecular Devices, Sunnyvale, CA, USA).

Isolation of human SC. Pooled samples of human milk (a gift from Ullevaal University Hospital, Oslo) were stored at −20 °C. Whey was obtained as described [18, 19]. Briefly, thawed milk was acidified to pH 4.6 with 1.0 M acetic acid and centrifuged for 30 min at 27 000 *g*, at 4 °C. The top fatty layer was gently removed, and the supernatant containing SC neutralized to pH 7. Then, ammonium sulphate was added to 70% saturation, and the mixture was rotated for 30 min in RT, followed by centrifugation at 5000 × *g*. The precipitate was dissolved in PBS/azid and dialysed against PBS overnight. A CNBr-activated Sepharose™ 4B column (10 ml) was coupled to IgM according to the manufacturer's recommendation and calculated to contain 30 mg IgM/ml resin. The SC containing fraction was passed through the column at an elution rate of 1.0 ml/min and the column washed with PBS. Finally, SC was eluted with 1.0 M KSCN dissolved in PBS pH 7.3. The eluted peak fractions were dialysed against PBS/azid and concentrated.

Lactoferrin (Lf) present in human whey interacts with the IgM Sepharose [19, 20], and copurified Lf was removed by affinity chromatography on a 5-ml HiTrap™ Heparin–Sepharose HP column (GE, Uppsala, Sweden). The column was equilibrated with binding buffer containing 0.05 M Tris/HCl, 0.2 M NaCl, 2 mM EDTA and 0.02% azid, pH 7.6, at a flow rate of 0.5 ml/min.

Isolation of IgM J+ and IgM J− fractions. Cell supernatant from IgM-producing transfectants was passed through the column containing SC. The unbound fraction (IgMJ-) was eluted by PBS, and bound material (IgMJ+) was eluted with 0.1 M KSCN in PBS followed by 0.5 M KSCN in PBS. IgM fractions were isolated from vaccine sera by passing the sera through the SC-column in a similar way.

ADCML assay. The ADCML studies were performed as previously described [21]. Briefly, ^{51}Cr-labelled sheep red blood cells (SRBC) were loaded with NIP-conjugated rabbit anti-SRBC Fab fragments, with an average of 4–60 NIP molecules per Fab fragment. The total amount of NIP introduced to the surface of 1×10^8 SRBC was 80 ng or 2000 ng, depending on the amount of Fab introduced. The complement serum was diluted 1:30 before portions were added to the target cell suspension of about $2–3 \times 10^7$ cells/ml. Serial dilutions of isolated IgM preparations were then added. The cytotoxic index (CI) was calculated according to the formula: $\%CI = ((\text{cpm test} - \text{cpm spontaneous})/(\text{cpm max} - \text{cpm spontaneous})) \times 100$.

ELISA measurements of complement activation. Complement activation was measured in ELISA using microtiter plates coated with $NIP_{15}BSA$ or meningococcal outer membrane vesicles (OMV) from group B meningococcal strain H44/67 and developed with anti-C1q anti-C3 or anti-C5 [22, 23]. The coated ELISA plates were washed with PBS pH 7.3 with 0.05% Tween-20 (PBS/T) and incubated for 1.5 h at 37 °C with serial dilutions (30–3000 ng/ml) of IgM solutions in PBS/T. After washing the plates, portions of 100 μl of a 1:200 dilution of human serum used as complement source (stored at −70 °C) in 0.1 M Veronal

buffer containing 0.25 mM $CaCl_2$ and 0.8 mM $MgCl_2$ pH 7.2 were added, and the plates incubated at 37 °C for 30–45 min. Then, the plates were washed, and a 1:4000 dilution (in PBS/T) of rabbit anti-human C5 (Dako, Copenhagen, Denmark) or a 1:10,000 dilution of rabbit anti-human C3c or C1q (Dako) was added and the plates incubated for 1.5 h at 37 °C. After washing, portions of biotin-labelled, locally produced sheep anti-rabbit IgG, biotin-labelled alkaline phosphatase and streptavidin were added and the plates were incubated for 1.5 h at 37 °C, before washing, incubation with the NPP substrate and absorbance 405 nm reading after various times of incubation.

Results

Isolation of IgM J+ and IgM J− fractions

IgM J+ and IgM J− fractions were isolated by affinity chromatography on a column containing human SC. The IgM J− molecules were eluted in the run-through fraction because these molecules cannot bind to SC. To ensure purity and remove any contaminating IgM J+ molecules, the procedure was repeated. The IgM J+ molecules were eluted from the SC-column by 0.5 M KSCN pH 7.0 after a prewash with 0.1 M KSCN pH 7.0, as this will distort the binding between the IgM J+ molecules and the SC-matrix (fraction III, Fig. 1). The polyclonal IgM formed after primary immunization of human volunteers was depleted for IgG antibodies which would otherwise interfere with the complement activation assay, by passing fractions of interest through a protein G column and elution with PBS. This fraction did not contain IgG (data not shown) and was further purified on the SC column.

The IgM J+ fractions bind SC and the IgM J− fraction does not

The two IgM fractions, one binding and one not binding to the SC-column, were tested for ability to bind free SC in an ELISA assay. Microtiter plates were coated with antigen, either $NIP_{15}BSA$ or OMV, the main constituent of the MenBVac® vaccine, washed with PBS and a constant amount of IgM was added followed by incubation for 2 h at 37 °C and the addition of serial dilutions (from 10 μg/ml) of purified SC. The ELISA results showed that the NIP-specific IgM J+ fraction could bind free SC, while the IgM J− fraction could not (Fig. 2). Similar results were achieved using polyclonal IgM specific for group B meningococci isolated from sera of volunteers receiving the MenBVac® vaccine (data not shown).

Complement activation measured by ELISA

We then added titrated amounts of SC to purified J+ and J- recombinant anti-NIP IgM as well as IgM fractions purified from sera and tested for complement activation by ELISA, using wells coated with $NIP_{15}BSA$ or OMV. All wells containing anti-NIP IgM were developed with antibodies specific for C1q, C3c or C5, respectively, to reveal complement activation at the level of C1q, C3 and C5. The results showed that the IgM J+ and the IgM J− fractions activated complement regardless of the addition

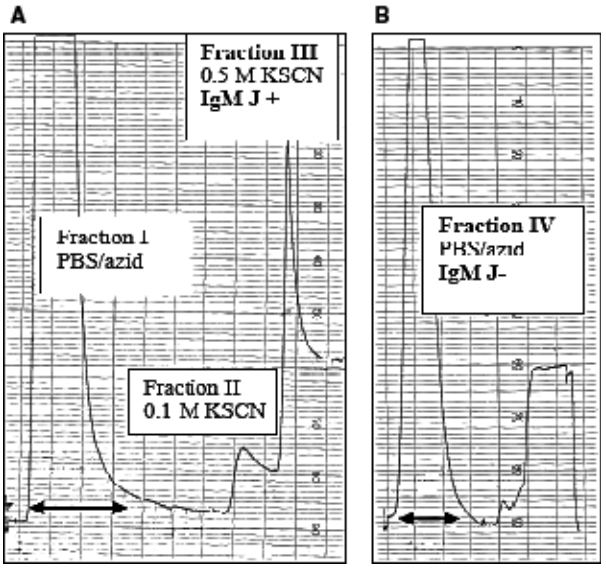

Figure 1 Isolation of IgM J+ and IgM J− fractions using affinity chromatography on Sepharose coupled with SC purified from human milk. 300 ml of cell supernatant from J558L-producing human chimeric NIP-specific antibodies was eluted through a 10 ml column containing 30 mg SC. (A) After applying the sample, the column was rinsed with 30 mg SC PBS/azid (fraction I) and then with PBS containing 0.1 M KSCN (fraction II) and finally with 0.5 M KSCN (fraction III). (B) After regeneration of the column, fraction I was run through the column again, resulting in a fraction (fraction IV) devoid of J-chain.

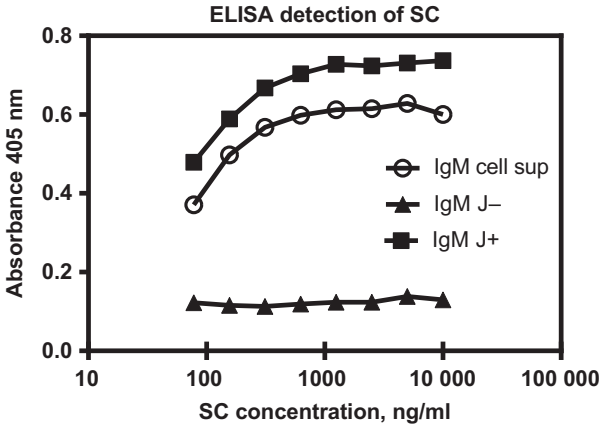

Figure 2 Detection of SC binding capacity of unpurified cell supernatant and fractions III and IV from Fig. 1. The three samples were tested at an equal concentration of IgM (1 μg/ml). Twofold increasing concentrations of SC, from 78 ng/ml to 10 μg/ml, were added and incubated for 30 min at 37 °C, before adding to microtiter plates coated with $NIP_{16}BSA$. The plates were developed with a polyclonal rabbit anti-human SC antiserum.

of SC (Fig. 3 A–C). Wells containing IgM formed after primary immunization with the meningococcal MenBVac® vaccine were developed with antibody specific for C3 and confirmed the result (Fig. 3D). There was a supple difference between the monoclonal anti-NIP antibodies and the polyclonal anti-OMV antibodies as the polyclonal IgM J− activated C3 more efficiently than IgM J+ (Fig. 3D), while no difference were revealed between monoclonal IgM J− and IgM+J+ (Fig. 3B). In neither case did the addition of SC influence the activation.

Antibody-dependent complement-mediated cell lysis (ADCML)

We then performed an ADCML analysis of the NIP-specific IgM J+ and IgM J− antibodies, where we tested the effect of antigen concentration and patchiness on target cells, complement source as well as antibody affinity. The results are recorded as lysis of sheep red blood cells. We found that the IgM J− fraction was more active in ADCML than the IgM J+ fraction especially at low antigen concentration (Fig. 4). The difference in ADCML of IgM J+ (less active) and IgM J− (most active) was moderate when human serum was used as complement source, but pronounced with guinea-pig serum (Fig. 4). When the complement source was changed to rabbit serum, the differences between IgM J+ and IgM J− disappeared (Fig. 5).

Influence on ADCML activity by adding SC to IgM J+ and IgM J−

We then added a fixed amount of SC (200 ng/ml) to variable concentrations of IgM J+ or IgM J− fractions and observed a slight decrease in the ADCML activity of IgM J+ (Fig. 4), regardless of complement source. The addition of 200 ng/ml of SC might have been suboptimal for

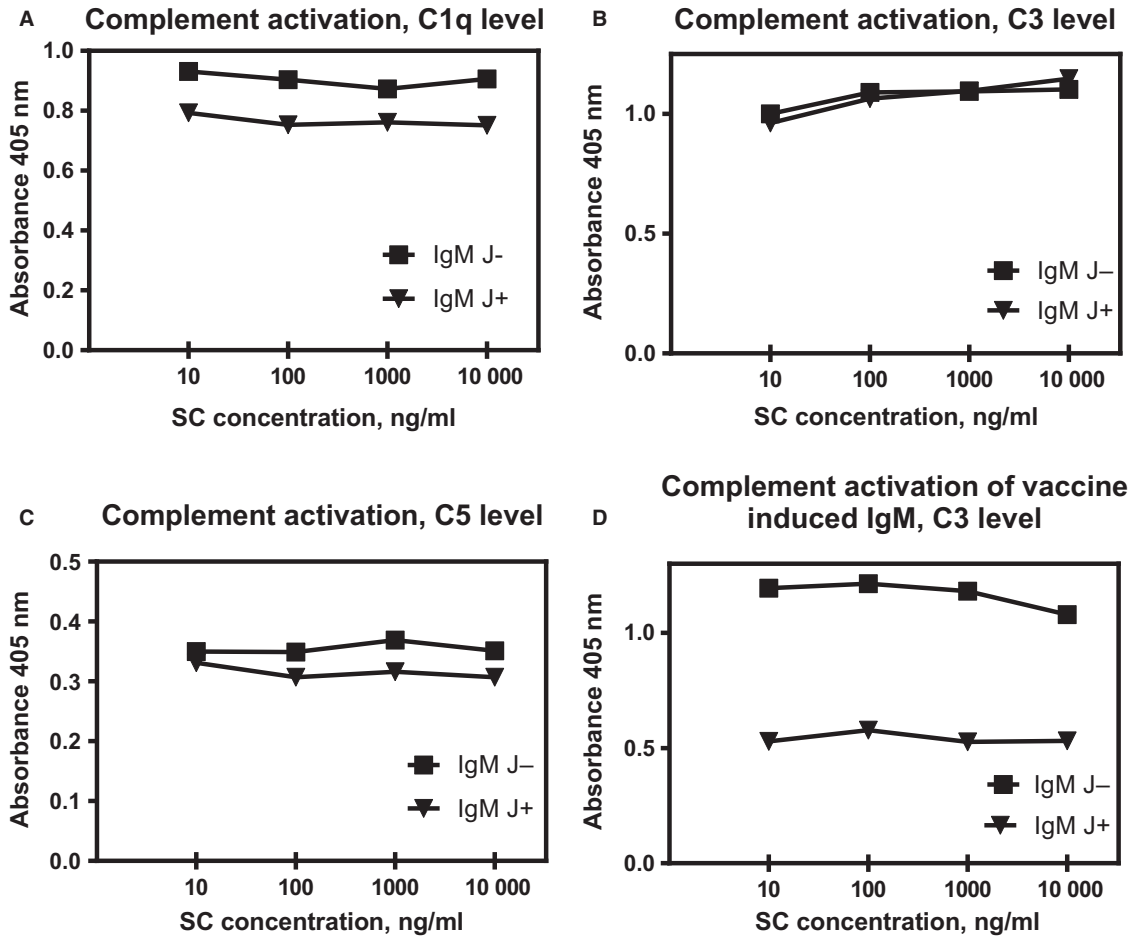

Figure 3 Complement activation activity measured in ELISA. Microtiter plates were coated with $NIP_{16}BSA$ (A, B and C) or meningococcal OMV (D), and an equal concentration of IgM (1 μg/ml) was added, followed by twofold increasing concentrations of SC, from 78 ng/ml to 10 μg/ml. After incubation and washing, an optimal dilution of human serum as complement source was added, and the plates incubated for 45 min at 37 °C, washed and developed with anti-C1q, C3c or C5 antiserum. For the wells coated with meningococcal OMV, a 1:200 dilution of human serum devoid of IgG was used as complement source [36].

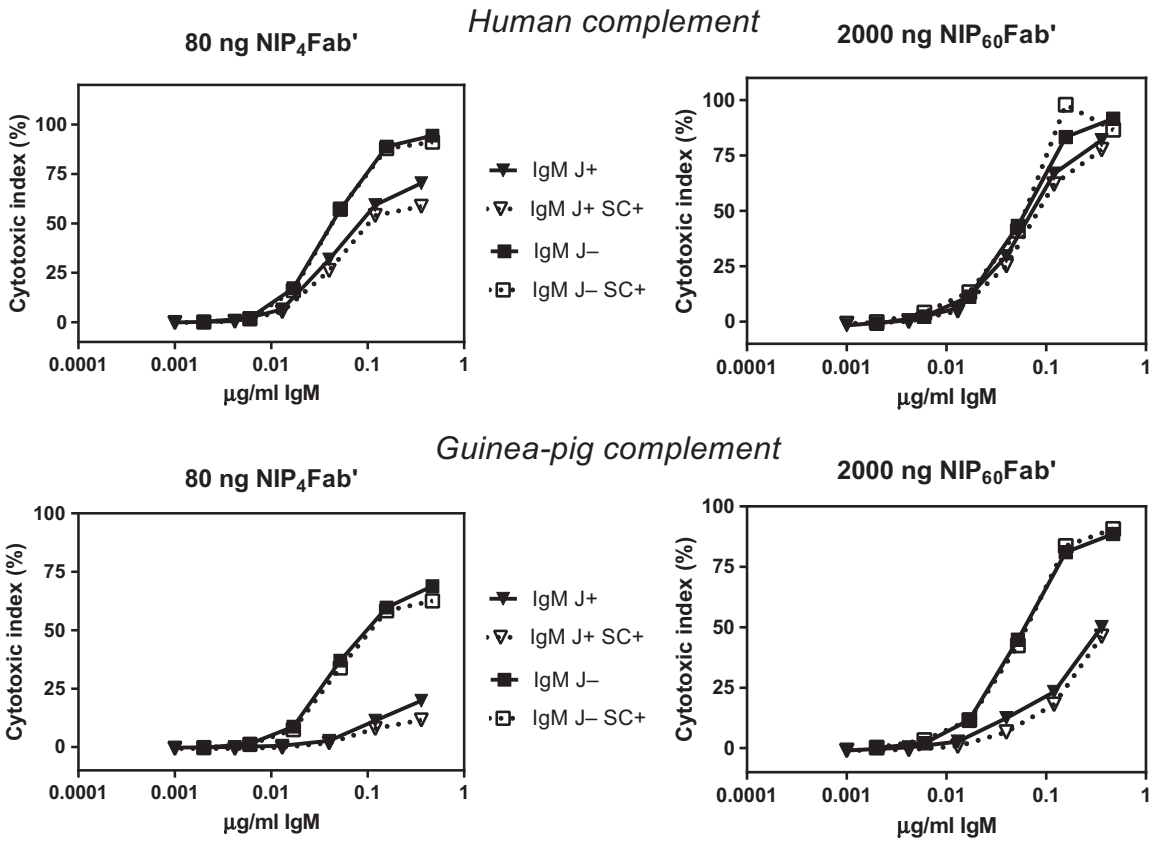

Figure 4 ADCML of IgM J+ and IgM J− fractions, with and without addition of 200 ng/ml SC, using human serum (upper panels) or guinea-pig serum (lower panel) as complement source. The antibodies were tested at low and high antigen concentration as well as low and high patchiness on the target cells.

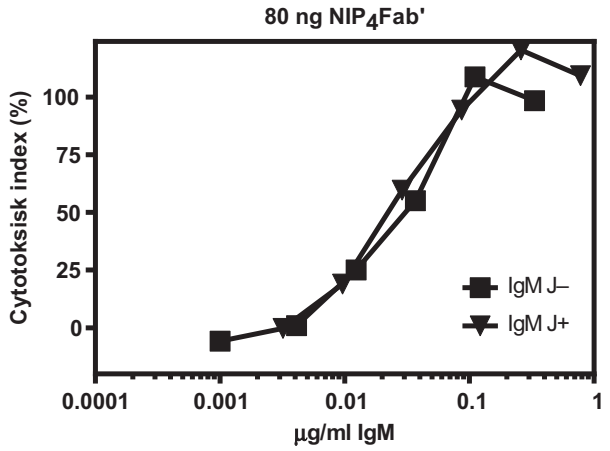

Figure 5 ADCML of IgM J+ and IgM J− fractions using rabbit serum as complement source. The antibodies were tested at low antigen concentration and low patchiness on the target cells.

creation of SIgM, and we also tested a fixed concentration of IgM (1 μg/ml) and added increasing amounts of SC (4–512 ng/ml), showing that SC lowered the ADCML activity only slightly, but apparently also the activity of the IgM J+ fraction (Fig. 6).

Discussion

IgM is present in blood mainly as pentamers [24], but variable amounts of hexamers may also exist [25]. J-chain present in polymeric IgA and pentameric IgM was first thought to be crucial for polymerization [25], but the identification of hexameric IgM devoid of J-chain challenged this notion [26]. The main function of J-chain seems now to be a structural prerequisite for active transport of polymeric IgA and IgM through the mucosal epithelium by the use of pIgR [4].

When IgM is secreted at mucosal surface as SIgM, it consists of pentameric IgM with at least one J-chain and at least one copy of SC [27], in contrast to serum IgM which does not contain SC.

In this report, we elucidate whether SIgM activates complement, and if so, how efficient it is compared to serum IgM. The SIgM molecules might be less active in complement activation than serum IgM, if SC were interfering with the molecular mechanism behind IgM complement activation involving the 'star' to 'staple' conformational change of IgM upon binding to cell surface antigens [28]. SC is intimately, non-covalently bound to IgM and lacks some

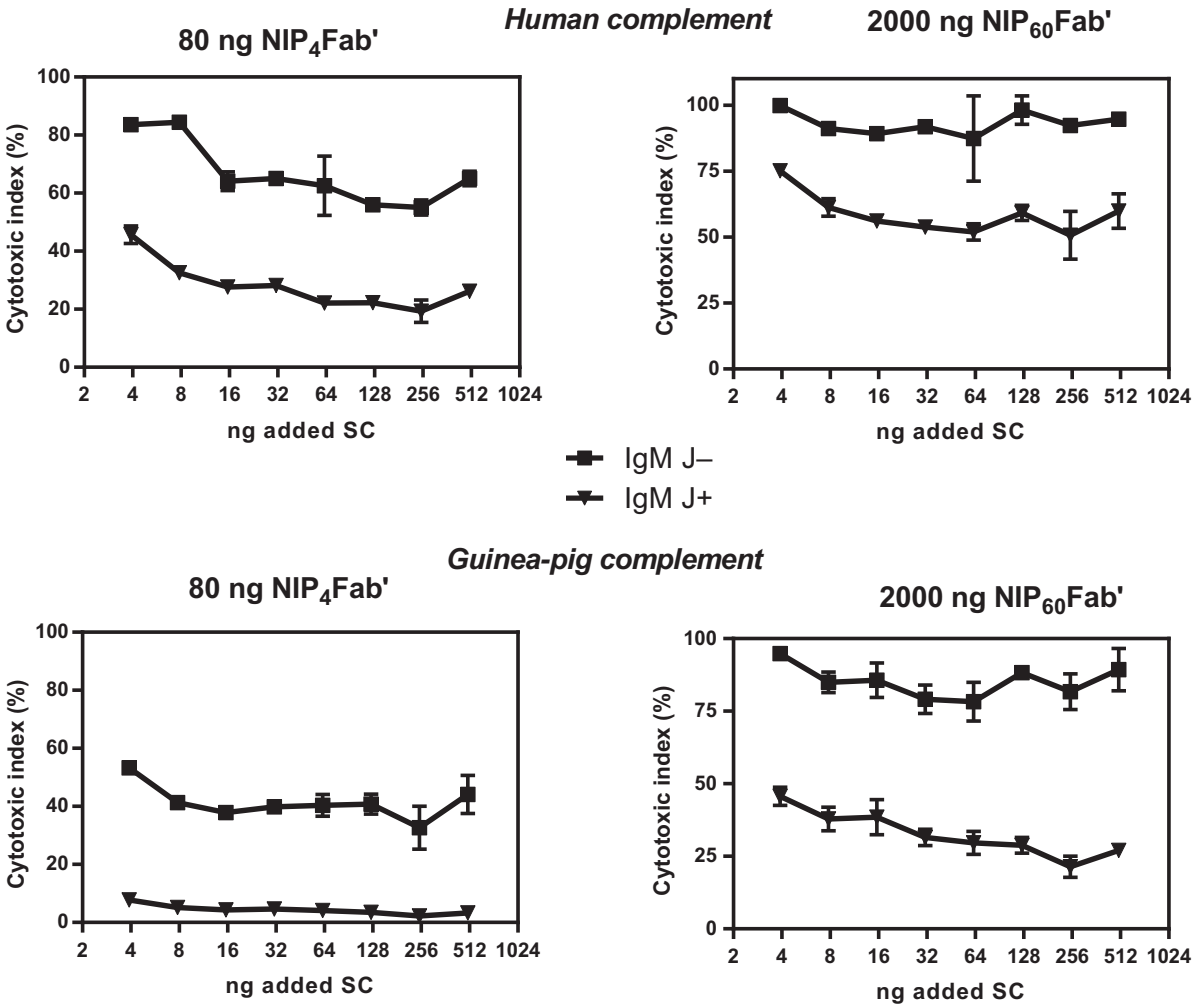

Figure 6 ADCML of IgM J+ and IgM J− fractions in the presence of twofold increasing concentrations of SC from 4 to 516 ng/ml. Human (upper panel) or guinea-pig (lower panel) serum was used as complement source. The antibodies were tested at low and high antigen concentration as well as low and high patchiness on the target cells. The concentration of the IgM fractions was 1 μg/ml in all cases.

antigenic epitopes expressed on free SC [29] and could impair complement activation. Furthermore, if SIgM were devoid of complement activation ability, it would not induce mucosal inflammation, which might be considered beneficial for gut and mucosal homeostasis.

To study the complement activation capacity of SIgM, we utilized monoclonal chimeric IgM with specificity for the NIP hapten. This is a flexible system, which allows for variation in antigen/epitope concentration on target cells as well as antibody affinity by switching between NIP and the NP hapten which has 50 times lower affinity than NIP [21, 30, 31].

The monoclonal IgM anti-NIP antibodies have relatively high affinity due to V-regions that are derived from a mouse hybridoma and may not necessarily reflect the IgM immune response in humans. We therefore included polyclonal IgM formed after primary immunization with a meningococcal vaccine. Strikingly, we observed essentially the same results for the natural polyclonal vaccine induced IgM antibodies and the recombinant, chimeric, monoclonal IgM, and thus, the affinity difference in the two systems does not bias our results.

We generated IgM fractions by affinity purification on a column containing human SC isolated from colostrum. This procedure allowed us to generate two fractions, IgM J+ and IgM J−. We titrated SC onto these preparations. When using human serum as complement source, the reconstructed SIgM (IgM J+ SC+) molecules were almost as active as IgM J+ without SC, mimicking secretory IgM and serum IgM, respectively (Fig. 4). Thus, SIgM has the capacity to be inflammatory in terms of complement activation. As far as we know, this is the first report of complement activation capacity of SIgM.

However, when using guinea-pig serum as complement source, human IgM J+/IgM J+ SC+ were weak in complement-mediated cell lysis, especially at low antigen concentration on the target cell. This resembles a previous report [8], showing weak induction of complement activation using guinea-pig serum and pentameric human IgM. Our results

could serve as a warning in the interpretation of old data involving guinea-pig complement and human antibodies. Historically, guinea-pig sera have been widely used as complement source in biological assays involving human antibodies. The reasons are probably high stability and concentration of complement factors in guinea-pig sera and also low assay background due to low concentrations of interfering cross-reacting antibodies in guinea-pig sera.

Our finding that SIgM activates human complement would also predict that SIgM at mucosal surface could induce efficient opsonophagocytosis of bacteria and other infectious agents. We have previously shown that monoclonal chimeric IgM with specificity for meningococcal PorA antigen is more efficient in inducing opsonophagocytosis of meningococci compared to chimeric IgG1 and IgG3 with identical V-regions [3]. Thus, IgM antibodies present on mucosal surface or in breastmilk would be expected to efficiently eliminate meningococci and other bacteria/pathogens in the presence of complement and/or phagocytes like neutrophils. Surprisingly, the anti-meningococcal IgM antibodies were less efficient than IgG1 and IgG3 in mediating complement-mediated bacteriolysis [3]. This is suggesting that IgM induces the formation of more C3 splits product deposits on the target cells than IgG. If this is also the case for SIgM, then it remains to be studied. Similar SIgM made from plasma IgM by adding recombinantly produced human SC have recently been published [14, 32] and these SIgM antibodies show protection against epithelial cells infected with an enteropathogen. However, this SIgM preparation was not tested for complement activation ability. Interestingly, SIgM showed better protection than parallel SIgA [32].

Complement concentration on mucosal surfaces is probably limited, although sufficient to induce activation and inflammation [13], while phagocytic leucocytes can be abundant at mucosal surface (for review see [33]).

The SC is also an integral part of SIgA, and SC probably does not interfere with SIgA binding affinity towards FcαRI, although this is controversial [34, 35].

Thus, neither J-chain nor SC seems to play any striking role in regulating IgM (and possibly IgA) effector functions apart from their obvious role in cross-mucosal transport of IgM as well as IgA.

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