Antigen Binding to Secretory Immunoglobulin A Results in Decreased Sensitivity to Intestinal Proteases and Increased Binding to Cellular Fc Receptors*

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In intestinal secretions, secretory IgA (SIgA) plays an important sentinel and protective role in the recognition and clearance of enteric pathogens. In addition to serving as a first line of defense, SIgA and SIgA-antigen immune complexes are selectively transported across Peyer’s patches to underlying dendritic cells in the mucosa-associated lymphoid tissue, contributing to immune surveillance and immunomodulation. To explain the unexpected transport of immune complexes in face of the large excess of free SIgA in secretions, we postulated that SIgA experiences structural modifications upon antigen binding. To address this issue, we associated specific polymeric IgA and SIgA with antigens of various sizes and complexity (protein toxin, virus, bacterium). Compared with free antibody, we found modified sensitivity of the three antigens assayed after exposure to proteases from intestinal washes. Antigen binding further impacted on the immunoreactivity toward polyclonal antisera specific for the heavy and light chains of the antibody, as a function of the antigen size. These conformational changes promoted binding of the SIgA-based immune complex compared with the free antibody to cellular receptors (FcαRI and polymer immunoglobulin receptor) expressed on the surface of premyelocytic and epithelial cell lines. These data reveal that antigen recognition by SIgA triggers structural changes that confer to the antibody enhanced receptor binding properties. This identifies immune complexes as particular structural entities integrating the presence of bound antigens and adds to the known function of immune exclusion and mucus anchoring by SIgA.

Mucosal surfaces comprising the gastrointestinal, respiratory, and urogenital mucosae represent a large port of entry for most of the pathogens and thus have to be efficiently protected. This task is achieved through a combination of constitutive and innate mechanisms relying on mucus, lysozyme, lactoferrin, defensins (1), and induced specific immunity based on the antibody (Ab)² level, on the production of secretory IgA (SIgA). In face of the large excess of SIgA in the intestinal lumen, intrinsic passage of the Ab or IC remains limited. In this context, we previously postulated that upon binding of the Ag, SIgA experiences conformational changes that result in increased binding to the so far unidentified IgA receptor on the apical surface of M cells (7). Conformational differences in plgA- or SIgA-based IC compared with the corresponding free Ab may also explain differential mucus-binding properties and immune exclusion (10). To address the question of Ag-mediated structural changes in plgA and SIgA, we focused our analysis on biochemical approaches comparing plgA and SIgA either as free Ab molecules or in association with three antigenic structures of increased complexity, namely a protein (Clostridium difficile toxin A), a virus (rotavirus), and a bacterium (Shigella flexneri).

In this study, we found that Ag-driven conformational changes can be evidenced by examining the differential sensitivity to intestinal proteases and immunoreactivity of IC compared with free Ab. Increased selective binding to known cellular receptors (FcαRI, plgR) further revealed major changes in IC compared with free Ab. The data support the notion that binding to Ag of various sizes affects the structure of plgA and polymeric Ig receptor; SC, secretory component; hSC, human SC; mSC, mouse SC; SIgA, secretory IgA.
S IgA molecules in such a manner that it makes Ag-complexed Ab molecules a better substrate for receptors that relay the function of the Ab.

EXPERIMENTAL PROCEDURES

Source of Protein—Chimeric IgAPCG-4 specific for C. difficile toxin A was produced as described (11). Ascites fluid (30 ml) was obtained from BALB/c mice inoculated intraperitoneally with S. flexneri serotype 5a lipopolysaccharide (16), was grown in Luria-Bertani medium. Bacteria grown overnight as a lawn were recovered in 0.9% NaCl, and their concentration was determined by spectrophotometry (Ultrospec 3000; Pharmacia Biotech) using the following correlation: 5.3 A 260 corresponds to 1 mg/ml virus.

Formation of IC—IC were formed as follows. 100 ng of C. difficile toxin A was mixed with 50 ng of either pIgAPCG-4 or S IgA permitting neutralization in in vitro assays (11). 60 ng of purified rotavirus was incubated with 100 ng of either pIgAC5 or SIgAC5 in PBS for 2 h at room temperature, corresponding to ~160 Ab molecules/virus (19). 106 S. flexneri were incubated with 120 ng of either pIgAC5 or SIgAC5 in PBS for 25 min on ice, corresponding to ~2000 Ab molecules/bacterial (20). These conditions guarantee that no free pIgA/S IgA are found in the IC preparations.

Digestion of Ab and IC with Mouse Intestinal Washes—A laparotomy was performed on BALB/c mice (4–6 weeks old) purchased from Harlan (Den Horst, The Netherlands). The gut was cut, and the intestinal lumen was washed with 300 µl of PBS. Final aspiration resulted in the recovery of ~200 µl of intestinal wash, which was immediately aliquoted and frozen in liquid nitrogen (6). For in vitro digestion, 120 ng of purified pIgA or S IgA (pIgAPCG-4, S IgAPCG-4, pIgAC5, SIgAC5, pIgACD9, SIgACD9) or IC was mixed or not with 1 or 2 µl of intestinal washes in a final volume of 20 µl of PBS and incubated at 37 °C for either 3 or 21 h. Reactions were immediately stopped by the addition of 2 µl of Complete™ protease inhibitor mixture (Roche Applied Science).

Western Blot Analysis—In Fig. 1, up to 400 ng of purified pIgA or S IgA was mixed in a final volume of 20 µl with SDS-sample buffer (100 mM Tris base, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) containing 100 mM dithiothreitol (Applichem, Darmstadt, Germany). Samples were heated to 95 °C for 3 min and subjected to electrophoresis in 6% polyacrylamide gels. For digests originating from the free or Ag-bound pIgA or S IgA, 40 ng (α chain detection) or 80 ng (κ chain detection) per lane was separated onto 10% or 18% polyacrylamide gels, respectively. After transfer onto polyvinylidene difluoride membranes (Millipore) and blocking for 1 h in PBS and 0.05% Tween 20 (PBS-T) containing 5% nonfat dry milk, proteins were detected by incubation for 1 h with the following Ab or combination of primary and secondary Ab in PBS-T 0.5% nonfat dry milk: goat anti-human α chain IgG conjugated with horseradish peroxidase (1/3000; Sigma); goat antimouse IgA diluted 1/3000 (α chain-specific; Sigma) and rabbit anti-goat IgG conjugated to horseradish peroxidase (1/3000; Sigma); rabbit anti-human κ chain antiserum (1/2000; Dako, Glostrup, Denmark) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1/3000; Sigma); goat anti-mouse κ chain IgG conjugated to horseradish peroxidase (Exalpha Biologicals, Watertown, MA); rabbit anti-hSC diluted 1/3000 (15) or rabbit anti-mSC diluted 1/3000 (14) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. After washing with PBS-T, membranes were developed with appropriate second-
ary Ab coupled to horseradish peroxidase. Proteins on membranes were detected by chemiluminescence using Uplight detection kit (Interchim, Montluçon, France) and exposed on autoradiographic films (Konica, Hevac Product, Villeneuve, Switzerland).

**Enzyme-linked Immunosorbent Assay (ELISA)**—96-well plates (Nunc-immuno Plate Maxisorp surface; Nalge Nunc International, Roskilde, Denmark) were coated with a range of 20–40 ng of either Ab, IC, or Ag in a final volume of 100 μl of coating buffer (PBS or NaHCO₃ (pH 9.6)) for 2 h at room temperature. After washing three times with PBS-T, wells were blocked with PBS-T containing 1% of bovine serum albumin (Fluka, Buchs, Switzerland) at 1 h at room temperature. Detection of human or mouse α- and κ chains was carried out for 1 h at room temperature with 100 μl/well of the same primary/secondary Ab/antiserum in PBS-T and 1% bovine serum albumin used in Western blot assays. After final washes, revelation was performed with a 0.1 M citrate sodium solution (pH 5.0) containing 1 mg/ml o-phenylenediamine (Sigma) and 0.01% H₂O₂. The reaction was stopped by the addition of 2 M sulfuric acid, and the absorbance was measured at 492 nm with 620 nm as reference.

**Cells**—The human promyelocytic cell line U937 was obtained from the American Type Culture Collection. The cells were plated in 75-cm² plastic flasks containing RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin, at 37 °C in a humidified 5% CO₂/air incubator. The cells were subcultured every third day, at a density of 3 × 10⁵ cells/ml. 18 h prior to binding experiments, cells were stimulated with 10⁻⁸ M phorbol myristate acetate because this was shown to increase surface expression of FcαRI (22). Transfected Madin-Darby canine kidney (MDCK) cells overexpressing the human plgR were cultured to 90% confluence on 6-well plastic plates (Nunc-immuno Plate Maxisorp surface; Nalge Nunc International, Roskilde, Denmark) and the absorbance was measured at 492 nm with 620 nm as reference.

**Flow Cytometry**—U937 or MDCK cells were washed twice with PBS, 2 × 10⁵ cells in 100 μl of PBS containing 1% fetal calf serum (PBS-S) were preincubated with 50 μg/ml human or mouse IgG for 30 min at 4 °C to mask FcγRs. After washing with cold PBS, cells were incubated for 1 h at 4 °C with 100 μl of PBS-S containing 2 μg of fluorescein-labeled plgA or SlgA or the same amount of IgA in the form of IC thereof. Unbound Ab or complexes were washed three times with ice-cold PBS. Cells were gently detached with a rubber policeman, allowing for viability above 90%, as determined by trypan blue staining. For binding inhibition studies, U937 cells were preincubated with 1 μg of anti-FcαRI mAb MIP8a (AbD Serotec, Düsseldorf, Germany), and MDCK cells with 10 μl of rabbit antiserum to hSC prior to the addition of fluorescent IgA or IC. Cell-associated fluorescence intensity was evaluated by flow cytometry (FACScan flow cytometer; Becton-Dickinson).

**Measurement of Binding Affinity**—U937 and MDCK cell-associated radioactivity was determined. Moles of bound plgAPCG-4, SlgAPCG-4, or IC formed with the toxin A were calculated, and equilibrium dissociation constants (Kₐ) and number of binding sites per cell were determined by Scatchard analysis (24).

**RESULTS**

**Proper Assembly of plgA and SlgA for Biochemical and Binding Studies**—To examine the consequences of the binding of Ag of various nature (protein, virus, bacterium) on IgA structure, we generated cognate monoclonal Ab in the two molecular forms relevant for mucosal immunity, i.e. plgA and SlgA. Purified plgA (IgAPCG-4, IgA7D9, and IgAC5) was analyzed by immunodetection following separation on polyacrylamide gel under nonreducing conditions (Fig. 1). Proper assembly and integrity of the molecules were checked using antisera specific for human α chain (lanes 1), the J chain (lanes 2), and reassociated SlgAPCG-4 assayed with anti-hSC antiserum (lanes 3). Mouse plgA7D9 (lanes 4 and 5), plgAC5 (lanes 7 and 8), and SlgA forms (lanes 8 and 9) were revealed using antisera specific for mouse α chain, J chain, and SC, respectively. The molecular mass (kDa) of immunoreactive species is shown alongside the lanes. In lanes 3, 6, and 9, the band at 80 kDa represents free SC not covalently bound to plgA.

**Statistical Analysis**—All statistical analyses were performed using GraphPad Prism version 5. For all data, an unpaired Student’s t test was applied, and the limit of significance was set at p = 0.05.
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**FIGURE 2.** *In vitro* digestion pattern and changes in ELISA reactivity of IgAPCG-4 molecules bound or not to their cognate antigen. A, comparative digestion pattern after a 3- or 21-h incubation with intestinal washes of plgAPCG-4 and SlgAPCG-4, complexed or not with *C. difficile* toxin A, assayed by immunodetection of the α chain. The intact α chain is detected as a single band of 62 kDa as shown on the right panel. B, same samples as in A, with immunodetection carried out with anti-κ chain antiserum. The single band at 24 kDa corresponds to intact κ chain. In A and B, the numbers just above the panels indicate the duration of digestion performed at 37 °C; one representative experiment of three is depicted. No cross-reactivity of the detection Ab and toxin A is observed (right lanes). C, change in α chain and κ chain reactivity of plgAPCG-4 or SlgAPCG-4 molecules in the absence or presence of toxin A, respectively. Data are expressed compared with the absorbance mean values obtained for plgAPCG-4 fixed arbitrarily at 100. Bars represent mean values ± S.D. (error bars) (*n* = 4). Significant statistical differences are indicated above the bars.

*vitro* degradation by intestinal washes mimicking the protease-rich environment found *in vivo* (6). We used the same technical approach to evaluate the impact of *C. difficile* toxin A binding on the sensitivity of plgAPCG-4 and SlgAPCG-4, as reflected by the appearance of α and κ chain degradation products assayed by immunodetection (Fig. 2, A and B). In time course experiments, the susceptibility of SlgAPCG-4 to intestinal washes was reduced compared with plgAPCG-4, with integral α chain (62 kDa band) recovered after 21 h of incubation in addition to degradation products of 40 and 36 kDa (Fd fragment). Upon association with the toxin A Ag, the pattern of digestion of the two molecular forms of the Ab was the same, with undegraded α chain detected at the 21-h end point. The C-terminal cleavage of the α chain, yielding the 36- and 40-kDa fragments, was shown to occur upon incubation with intestinal washes (6), some distance away from the Fab domains. This suggests that the susceptibility of the protein is reduced through the preferential action of conformational changes rather than masking of sensitive sites. In contrast, the integrity of the κ chain was not affected under any experimental conditions, arguing intramolecular and toxin A-mediated steric hindrance ensuring protection against the action of proteases (Fig. 2B).

Changes in conformation and topography in plgA and SlgA molecules have been assessed by analyzing the immunoreactivity of the constituting polypeptides in the Ab (26). Hence, we compared the reactivity of free and toxin A-bound plgAPCG-4 and SlgAPCG-4 with antisera specific for either the α or the κ chains by ELISA (Fig. 2C). We chose to use polyclonal antisera to permit to map the overall effect of toxin A binding on the availability of linear and conformational epitopes. For both chains, a weak (16–18%) yet highly reproducible reduction in reactivity was detected upon toxin A binding. The effect was slightly reinforced in the presence of SC in the Ab molecule (23–25% reduction), possibly accounted for by the capacity of SC to bind with toxin A (19) or masking of epitopes on the α chain. Together, this reflects changes directly associated with the impact of the bound Ag. Furthermore, the assay is complementary to that based on exposure to protease because it also implicates the κ chain in the process.

**Impact of a Whole Virus Ligand on IgA Structure**—We next investigated whether the variability in protease sensitivity and immunoreactivity of plgA/SlgA we observed after association with a protein Ag would similarly occur in the context of Ag of much bigger sizes and thus epitopic complexity. In comparison with the "naked" Ab that remained degradable into low molecular mass α chain products, incubation of rotavirus with VP6 (surface-exposed virus protein 6)-specific plgA7D9 or SlgA7D9 led to the demonstration that the Ab in either molecular form was almost fully protected against the action of proteases in intestinal washes (Fig. 3A). However, for this particular IgA, the addition of SC did not offer a better protection against proteases; this might be due to the low percentage of covalent interaction between SC and the α chain, or alternatively, to the intrinsic stability of IgA7D9 in the assay (Fig. 1). Together, these features may partially mask the impact of Ag binding in this assay. In support of a less reduced protective effect of SC associated with IgA7D9, reduction of the κ chain signal was observed after 21 h of incubation both in the absence and in the presence of SC. Tight interaction with globular rotavirus in a Fab-dependent manner maintained a stable level of κ chain comparable between lanes (Fig. 3B).
A clearer picture could be drawn from the immunoreactivity assay. Compared with IgAPCG-4, more marked changes in the capacity to be recognized by a chain-specific antiserum were measured for plgA7D9 (30% reduction) and S IgA7D9 (34%) (Fig. 3C). The decrease in α chain reactivity can be explained by reduced access to the IgA-associated epitopes as well as by conformational effects limiting productive interactions with the antigen, both resulting from coating of the virus particle by the Ab. In plgA7D9, κ chain in close proximity with the virus Ag displayed a reduction in reactivity (18%), favoring the masking hypothesis over distant structural changes in this case. In S IgA7D9, the drop in immunoreactivity reached 22%, in support of the spatial distribution of SC wrapping the constant domains of the protein (27).

Impact of a Whole Bacterium Ligand on IgA Structure—Comparison of free plgA/SIgA and the corresponding IC made of a protein or a viral Ag indicates that close interaction between partners on one hand and induced subtle modifications in the Ab structure on the other hand are differently affected as a function of the Ab-Ag couple. We further challenged this notion by extending our analysis to the effect of associating S. flexneri to IgAC5 specific for the bacterial surface lipopolysaccharide. Upon Ag binding, both molecular forms of the IgA Ab behaved the same, with a mixture of undigested α chain and 36- and 40-kDa degradation products detected at the two time points (Fig. 4A). This contrasted with the pattern obtained in the absence of S. flexneri, where unexpectedly, it appeared that pronounced degradation of the Ab took place at 21 h irrespective of bound SC (Fig. 4A). Although some cross-reactivity between the anti-α chain Ab and S. flexneri proteins could be observed, its contribution to the specific signal could be neglected in terms of interpretation of the results. The stability of the κ chain was demonstrated under all experimental conditions (Fig. 4B).

The immunoreactivity of the α chain was about the same between plgA and SIgA (Fig. 4C), suggesting a loose, although covalent association of SC, consistent with the limited protection observed in Fig. 4A. In contrast, a 26% reduction of κ chain reactivity occurred upon association with SC; this might result from a particular folding of the Fab domains in SIgAC5 because the first molecular model for SIgA shows surface exposure of two of four κ chains (27). The combination of the bacterium with the Ab led to a marked drop in α chain immunoreactivity for both the polymeric (91%) and secretory (92%) forms of IgAC5. A slightly less pronounced reduction for κ chain was measured for plgAC5 (81%) and SIgAC5 (86%). In complexes with S. flexneri, these changes could be caused by enhanced epitope density recognized by the IgAC5 Ab or, alternatively, by masking of proteolytic sites in the α and κ chain.

Binding of Free IgA and IC to Cellular Receptors—Results obtained by exposure to intestinal washes and immunoreactivity argue in favor of multiple consequences on the structure of the plgA/SIgA Ab molecules following interaction with Ag of various sizes and nature. At the biochemical level, these combined approaches led us to conclude on the dichotomy between structural changes and steric hindrance. To gain further insight into the impact of Ag binding on plgA/SIgA molecules, the interaction with two known receptors of the Ab was evaluated using cell lines expressing FcαRI or plgR. We focused our analyses on toxin A-plgA/SIgA IC because surface distribution of the fluorescent Ab on the rotavirus and S. flexneri Ag would bias the assessment of the mere contact between the receptor and the Ab ligand. For the duration of the experiment, we initially checked that toxin A assayed alone was not toxic for the
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![Figure 4](image-url)

**DISCUSSION**

The data reported herein demonstrate that, upon Ag binding, plgA and SlgA experience structural changes reflected in the differential sensitivity to intestinal proteases and immunoreactivity toward $\alpha$ and $\kappa$ chain-specific antisera. Furthermore, Ag binding increased the capacity of the Ab molecule to interact with two well documented IgA cellular receptors, namely Fc$\alpha$RI and plgR. Thus, the conformational changes characterized by epitope recognition, protease sensitivity, and binding to cellular receptor have physiologic consequences particularly relevant to mucosal immunology.

The nature and size of the Ag did not seem to be crucial parameters for these structural changes to occur because we found measurable effects when using a protein, a virus, or a bacterium in association with their cognate plgA/SlgA Ab. The formation of IC of higher size range due to an increasing number of anchoring sites present on the Ag did indeed reduce enzymatic degradation of the $\alpha$ chain, a phenomenon accompanied by a decrease in epitope recognition. In addition, structural “freezing” of the Ab molecule upon interaction with the Ag might as well explain the differences found with respect to sensitivity to proteases or diminished recognition by antisera. Because interaction with cellular receptors was improved, we believe that conformational changes mediated by Ag binding are principally responsible for the observed effects, with steric hindrance playing a limited role. Moreover, this is consistent with the more artificial situation where aggregated plgA exhib-
ited a better binding to target cells (28), possibly because of reduced dissociation of polymers as seen in our data and Ref. 29. In the context of IC, Ag recognition brings together Ab molecules and thus increases productive interactions with cellular receptors.

We reported in a previous study that IgA-based IC are taken up by intestinal M cells in Peyer’s patches (20). Although the receptor(s) involved is (are) in need of identification, the phenomenon is specific for the IgA isotype (mouse IgA and human IgA2 only) and requires both the FcγRI and FcγRII domains of the Ab (7). In face of the large excess of free SIgA in mucosal secretions, one would have expected that sampling by M cells is an unlikely event. We speculated that preferential uptake of SIgAAg complexes compared with free SIgA might be due to conformational change(s) that favor(s) binding to the M cell receptor. In support of our working hypothesis, we found that binding to Ag resulted in decreased sensitivity of the Ab to the action of proteases in the intestinal washes, indicating that domains in the Fc region were most likely in a more closed conformation or at least presented less accessible cleavage sites for intestinal enzymes. The effect was limited to the C-terminal portion of the Ab molecule because the κ light chain buried in the Fab was not, or only slightly, affected by the incubation with intestinal washes. An immunoreactivity study led to a decline in α chain and κ chain recognition upon Ag binding, and the effect was even more pronounced for SIgA, as expected from the wrapping of SC around the Fc domains of pIgA (27).

Increased interaction with pIgR and FcγRI of IC further indicates that important structural modifications occurred, which might also have relevance for the sampling of Ag by M cells. If we assume a multivalent nature for the interaction between pIgA/SIgA-based IC and the M cell receptor(s), this would lead to a slow off-rate, allowing stable binding and subsequent internalization, even in the presence of the high free SIgA concentrations found in exocrine secretions. The same mechanism has been postulated to explain FcγRI-mediated phagocytosis by monocytes in serum containing large excess of circulating IgA (30).

We observed identical association of free pIgA and SIgA with FcγRI on the surface of U937 cells. This confirms previous data of fluorescence is shown in the first four lanes, and binding specificity is confirmed by inhibition with the anti-FcγRI mAb MIP8a. B, same type of analysis performed with MDCK cells overexpressing human pIgR and fluorescein-la- beled pIgAPCG-4/SlgAPCG-4 associated or not with toxin A. Specificity of binding is controlled by inhibition of binding in the presence of anti-human pIgR antisera and by the absence of binding of SIgA molecular forms. In plots A and B, numbers indicate the mean of the cell-associated fluorescence intensity ± S.D. (error bars) of four to six individual experiments evaluated by flow cytometry. Significant statistical differences are indicated above the bars.

| Ligand | U937 cells Kd nM | MDCK cells Kd nM |
|--------|------------------|------------------|
| pIgAPCG-4 | 15.3             | 9.0              |
| SIgAPCG-4 | 17.7             |                  |
| pIgAPCG-4 + toxin A | 2.4          | 1.1              |
| SIgAPCG-4 + toxin A | 3.3          |                  |

**FIGURE 5.** Analysis of the binding capacity of pIgA/SIgA antibodies and immune complexes to cellular receptors. A, flow cytometry analysis of the binding capacity of fluorescently labeled pIgAPCG-4/SlgAPCG-4 complexed or not with the toxin A cognate Ag to U937 cells expressing FcγRI. Background level
showing binding of either form of the Ab, yet in a less quantitative manner (31). Although residues involved in the binding to FcαRI seem to also interact with SC (32–34), the expression of the co-receptor Mac-1 (CD11b/CD18, complement receptor 3) (35) on the surface of U937 stimulated with phorbol myristate acetate is sufficient to guarantee recognition of S IgA as well. In the form of IC, both pIgA and S IgA bound more efficiently to U937 cells, indicating that conformational changes most likely relayed by the Fab domains promoted interaction with FcαRI. This is consistent with structural variability mapped in FcαRI upon interaction with the recombinant Fcα fragment consisting of domains Cα2 and Cα3 (30). The importance of the Cα2/Cα3 junction as sites of functional diversity is indirectly reflected by the fact that it appears to be a hot spot for targeting of molecules produced by pathogens; such a mechanism of subverting immunity has been documented for Staphylococcus aureus SSL7 (36), group A streptococcus Sir22 (37), SSL7 (36), group A streptococcus Sir22 (37), and Staph-

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