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INTERACTION OF STAPHYLOCOCCAL PROTEIN A WITH MEMBRANE COMPONENTS OF IgM- AND/OR IgD-BEARING LYMPHOCYTES FROM HUMAN TONSIL[1]

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Human tonsil lymphocytes were examined for their ability to form rosettes with human red blood cells coated with staphylococcal protein A (SpA-HRBC). A significant proportion of cells from unseparated tonsil suspensions were able to form rosettes with SpA-HRBC. Purified T lymphocytes did not form rosettes with SpA-HRBC, whereas in T- cell-depleted suspensions the number of cells forming rosettes with SpA-HRBC was significantly greater than in unseparated populations. After depletion in cells forming rosettes with HRBC coated with anti-human γ-chain immunosorbent-purified rabbit antibodies, a noticeable proportion of B lymphocytes still showed the ability to form rosettes with SpA-HRBC. In addition, when tonsil lymphocyte suspensions were depleted in cells forming rosettes with SpA-HRBC, IgG-bearing cells were no more detectable, but the number of IgM- and of IgD-bearing lymphocytes was markedly reduced in comparison with that found in unfractionated populations. Finally, in tonsil suspensions depleted in IgM- and/or IgD-bearing lymphocytes, the number of cells forming rosettes with SpA-HRBC was significantly reduced in comparison with unfractionated populations and paralleled that of IgG-bearing lymphocytes.

The SpA-reacting components present on the membrane of some B lymphocytes were sensitive to treatment with low concentrations of pronase, but other B cells maintained the ability to form rosettes with SpA-HRBC even after treatment with higher pronase concentrations. The membrane components reacting with SpA-HRBC, which were sensitive to the treatment with pronase, were reexpressed by most lymphocytes after 18 hr in culture.

The incubation of tonsil lymphocytes with anti-human γ-, but also with anti-human μ- or anti-human δ-chain F(ab')$_2$ fragments induced a significant decrease in the number of rosettes formed by cells with SpA-HRBC. After incubation of cell suspensions with a mixture of anti-γ-, anti-μ-, and anti-δ-chain F(ab')$_2$ fragments, virtually no lymphocytes forming rosettes with SpA-HRBC could be found. On the other hand, SpA preincubation of lymphocytes significantly reduced the number of rosettes formed by cells with HRBC coated with either anti-human μ- or anti-human δ-chain F(ab')$_2$ fragments.

These data suggest that SpA interacts with IgG, but also with membrane components strictly related to IgM and/or IgD, present on the surface of a noticeable proportion of B lymphocytes from human tonsil.

Protein A of *Staphylococcus* (SpA)[3] has been shown to specifically bind to the Fc region of IgG immunoglobulin from several species (1, 2). This reaction was found to be suitable for detection and quantitation of human lymphoid cells carrying IgG, either at the cell surface as receptors or attached to the membrane as antibodies against surface antigens (3–5). More recently, it was shown that SpA-containing staphylococci are an efficient mitogen for human B lymphocytes with not detectable activity for T lymphocytes (6, 7) and are also capable of inducing polyclonal antibody secretion in human lymphocytes (8, 9). However, the exact nature of B cell subsets activated by SpA-containing staphylococci and the mechanism by which these bacteria exert their mitogenic activity are unknown.

We report here experiments undertaken to determine whether SpA is able to interact with human B cell surface components other than intrinsic or cytophilic IgG. This possibility was tested by evaluating the ability of lymphocytes from human tonsil to form rosettes with SpA-coated human erythrocytes.

MATERIALS AND METHODS

Staphylococcal protein A (SpA). Soluble SpA and SpA coupled to CNBr-activated Sepharose CL-4B were purchased from Pharmacia (Uppsala).

Anti-immunoglobulin (Ig) antisera. All anti-human Ig antisera were prepared in rabbit and purified with immunosorbents. For immunization, 2 mg of protein were emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, Mich.) and injected intramuscularly and into the foot pads. Rabbits were boosted 15 days later with intramuscular injections of 2 mg of protein emulsified in CFA, and 30 days later with intramuscular injections of 2 mg of protein emulsified in incomplete Freund's adjuvant (Difco). Antiserum specific for human IgM was prepared by injecting rabbits with IgM obtained from pooled sera of macroglobulinemia patients by euglobulin precipitation, fol-

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[3] Abbreviations used in this paper: SpA, staphylococcal protein A; HRBC, human red blood cells; E-RFC, E-rosette-forming cells; SpA-HRBC, human red blood cells coated with SpA; PBS, phosphate-buffered saline, pH 7.2.

lowed by Sephadex G-200 gel-filtration. Antiserum specific for human IgD was prepared by injecting rabbits with IgD isolated from the serum of a patient suffering from multiple myelomatosis by DEAE-cellulose chromatography in the presence of ϵ-amino caproic acid, followed by Sephadex G-200 gel-filtration (10). Antiserum specific for human IgG was prepared by injecting rabbits with IgG isolated from normal sera by DEAE-cellulose chromatography. Anti-human $F(ab')_2$ serum was obtained by injecting rabbits with human $F(ab')_2$ prepared by pepsin digestion from normal human IgG, and then purified by Sephadex G-150 gel-filtration and affinity chromatography with SpA-Sepharose CL-4B to remove undigested IgG (11). Human IgA was obtained from pooled sera of patients suffering from multiple myelomatosis by repeated gel-filtration on Sephadex G-200 and further purified by affinity chromatography with SpA-Sepharose CL-4B, to remove contaminating IgG (11). Human IgG, IgM, IgA, IgD, and $F(ab')_2$ were coupled on CNBr-activated Sepharose 4B (Pharmacia, Uppsala) (12). For the purification of anti-IgM (anti-μ) antibody, the anti-IgM serum was passed through an IgG, an IgA, and IgD, and a $F(ab')_2$ immunosorbent column to remove any contaminating antibody. The effluent was then passed over an IgM immunosorbent column. The column was washed extensively with phosphate-buffered saline, pH 7.2, (PBS) until the effluent contained less than 0.01 O.D. units at 280 nmoles. The antibody was then eluted with glycine-HCl buffer (0.1 M, pH 2.5) containing NaCl (0.5 M). The eluate was neutralized by direct filtration on a Sephadex G-15 column equilibrated with 0.15 M NaCl. For the purification of anti-IgD (anti-δ) antibody, the anti-IgD antiserum was passed through an IgG, an IgM, an IgA, and a $F(ab')_2$ immunosorbent column to remove any contaminating antibody. The effluent was then passed over an IgD immunosorbent column, and the antibody was isolated by acidic elution as described above. For the purification of anti-IgG (anti-γ) antibody, the anti-IgG antiserum was passed through a $F(ab')_2$, an IgM, an IgD, and an IgA column. The effluent was then passed over an IgG immunosorbent column, and the antibody was isolated by acidic elution as described above. Purified anti-$F(ab')_2$ antibodies were prepared by elution of the anti-human $F(ab')_2$ serum through the $F(ab')_2$ immunosorbent column, followed by acidic elution as described above.

$F(ab')_2$ fragments were prepared by pepsin digestion from anti-human γ-, anti-human μ-, and anti-human δ-chain immunosorbent-purified rabbit IgG, and then purified by Sephadex G-150 gel-filtration and affinity chromatography with SpA-Sepharose CL-4B (11).

Specificity tests. The specificity of all antisera was checked by double diffusion in agarose. In the case of anti-μ, anti-δ, and anti-γ antisera, the specificity was further checked by immunofluorescence on bone marrow smears from patients with IgG, IgD, and IgA myelomas and Waldenström's disease and by a paper disc radioimmunoassay. In this assay, antisera were bound to CNBr-activated paper discs according to the method of Ceska and Lundkvist (13). IgM, IgD, or IgG molecules were allowed to bind to antibody-coated discs. ^{125}I-radioiodinated, immunosorbent-purified antibodies were then allowed to react with the bound molecules. Anti-μ antiserum was found capable of reacting with IgM only, anti-γ antiserum with IgG only, and anti-δ antiserum with IgD only.

Coating of erythrocytes with anti-Ig and SpA. Immunosorbent-purified rabbit antibodies or SpA were coupled to human type O red blood cells (HRBC) with chromic chloride according to the technique described by Gold and Fudenberg (14). Briefly, 1 volume of washed and packed HRBC and 1 volume of immunosorbent-purified anti-human μ-chain, anti-δ-chain, anti-human γ-chain, anti-human $F(ab')_2$ rabbit IgG (1 mg/ml), anti-human μ-chain, anti-human δ-chain $F(ab')_2$ fragments prepared by pepsin digestion from rabbit IgG (1 mg/ml) or of SpA (1 mg/ml) in 0.15 M NaCl were mixed. Then, 1 volume of $CrCl_2 \cdot 6H_2O$ (1 mg/ml) in 0.15 M NaCl was added. The three components were mixed and allowed to react at room temperature for 5 min. The coated HRBC were washed three times with 0.15 M NaCl and resuspended to 1% in 0.15 M NaCl. Fresh preparations were made weekly.

Preparation and separation of tonsil lymphocytes. Palatin tonsils from unselected patients were collected under sterile conditions in cold TC 199 medium (Difco), supplemented with 1000 units/ml of penicillin G, 500 μg/ml of streptomycin, 100 μg/ml of kanamycin, and 1 μg/ml of gentamicin. Within 30 min after tonsillectomy, the tonsil was gently sliced in an ice bath and the tissue was dissociated by teasing. Large clumps of cells and tissue fragments were allowed to settle for 5 min, and the supernatant cell suspension was recovered and centrifuged on a Ficoll-Hypaque density gradient. The mononuclear cells recovered at the interface were incubated with carbonyl iron powder at 37°C on a rotator for 1 hr. Phagocytic cells were then removed by magnetism. The cells recovered were washed three times and used either directly or after various other purification procedures.

To obtain pure T lymphocytes, tonsil populations were rosetted with neuraminidase-treated sheep red blood cells (SRBC), as detailed elsewhere (7). E-rosette-forming cells (E-RFC) were separated from non-E-RFC by centrifugation on a gradient of Ficoll-Hypaque. The pellet was resuspended and further centrifuged on a second density gradient. SRBC were lysed by 0.87% NH_4Cl. Suspensions virtually free from T lymphocytes were obtained from tonsil populations by a double E-rosetting procedure, previously reported in detail (7). Briefly, non-T cells obtained from the interface after the initial density gradient separation of E-rosetting cells was deprived of residual T cells by rosetting again with neuraminidase-treated SRBC and density gradient separation. This double-step procedure gave suspensions consistently containing less than 1% E-RFC. Contaminating monocytes were identified by nonspecific esterase stain (15). The number of esterase-staining cells found in non-T cell populations obtained from tonsil suspensions initially depleted of phagocytic cells with carbonyl iron was consistently lower than 5%.

Rosette assay with anti-human Ig- or SpA-coated HRBC (SpA-HRBC) and Ficoll-Hypaque gradient separation of rosetted lymphocytes. One volume of the lymphocyte suspensions (2×10^6/ml) was mixed with one volume of 1% anti-human Ig- or SpA-coated HRBC and centrifuged at 200 × G for 5 min at 4°C. The pellet was resuspended by pipetting and stained with toluidine blue, and the percentage of rosettes was determined by microscopic examination of at least 300 cells.

In order to separate nonrosetted from rosetted lymphocytes, 1 ml of the rosetted mixture was layered on 2 ml of Ficoll-Hypaque and centrifuged at 400 × G for 30 min at 4°C. After centrifugation, the cells recovered at the interface were collected (16).

Enzymatic treatment of cells. Cells were resuspended at a concentration of 10×10^6/ml in TC 199 medium containing different amounts of pronase (Serva, Heidelberg), incubated at 37°C for 30 min, and washed three times in TC 199 medium supplemented with 10% fetal calf serum (FCS; Grand Island Biological Co., Grand Island, N. Y.).

Inhibition of rosette formation. Cells were resuspended at a

concentration of 10×10^6/ml in TC 199 medium. One-tenth milliliter of this suspension (1×10^6 cells) was incubated with varying amounts of SpA or of $F(ab')_2$ fragments prepared by pepsin digestion from immunosorbent-purified anti-human μ-, anti-human δ-, and anti-human γ-chain rabbit antibodies, in ice for 1 hr with gentle shaking at intervals. After two washings, the cells were tested for their ability to form rosettes with HRBC coated with SpA, anti-human heavy chain rabbit antibodies or their $F(ab')_2$ fragments, as described above.

RESULTS

Ability of unfractionated, T cell-depleted and T cell-enriched lymphocyte suspensions from human tonsil to form rosettes with SpA-HRBC. The capacity of unfractionated, T cell-depleted and T cell-enriched suspensions to form rosettes with SpA-HRBC is presented in Table I. A noticeable proportion of cells from unseparated tonsil suspensions (range 8 to 32; mean value 23 ± 3%) were able to form rosettes with SpA-HRBC. Table I also shows that these cells are B lymphocytes. In fact, purified T lymphocytes did not form rosettes with SpA-HRBC, whereas in T cell-depleted suspensions, where the number of Ig-bearing lymphocytes increased on the average from 54 up to 91%, the proportion of cells forming rosettes with SpA-HRBC increased on the average from 23 up to 48%. Rosette formation between tonsil lymphocytes and SpA-HRBC was inhibited if the cells were previously treated with soluble SpA. The minimum amount of SpA that was able to inhibit rosette formation to more than 50% was approximately 1 μg/10^6 cells.

TABLE I

Ability of unfractionated, T cell-depleted and T cell-enriched lymphocyte suspensions from human tonsil to form rosettes with SpA-coated HRBC[a]

Cell Population	% of E Rosettes	% of Rosettes using HRBC Coated with	
		Anti-$F(ab')_2$	SpA
Unfractionated	42 ± 3	54 ± 6	23 ± 3
T cell depleted	<1	91 ± 3	48 ± 6
T cell enriched	97 ± 2	<1	<1

[a] Results expressed as mean percentage ± S.E. of seven separate experiments.

Effect of depletion in IgG-bearing cells. In order to investigate the nature of B cell subsets reacting with SpA-HRBC, tonsil suspensions were rosetted with HRBC coated with immunosorbent-purified anti-human γ-chain rabbit antibodies. Nonrosetted were then separated from rosetted lymphocytes by centrifugation on a Ficoll-Hypaque density gradient. As shown in the Figure 1, the depletion in cells forming rosettes with HRBC coated with anti-human γ-chain antibodies resulted in an increase in the number of E-RFC and in a decrease in the number of Ig-bearing cells. The number of IgM- and IgD-bearing lymphocytes recovered at the interface of the gradient was unchanged in comparison with that of unfractionated population, whereas the number of cells forming rosettes with SpA-HRBC was decreased. However, in the interface populations, where no IgG-bearing cells could be found, a proportion of Ig-bearing cells ranging between 30 and 50% still showed the ability to form rosettes with SpA-HRBC. These data suggest that SpA is capable of binding to a remarkable subpopulation of cells that are apparently lacking of membrane IgG.

Effect of depletion in cells forming rosettes with SpA-HRBC. To obtain a further control of the capacity of SpA-HRBC to interact also with a B cell subset other than IgG-bearing lymphocytes, tonsil suspensions were rosetted with SpA-HRBC. Nonrosetted were then separated from rosetted lymphocytes by centrifugation of the cell mixture on a Ficoll-Hypaque density gradient and tested for their ability to form rosettes with HRBC coated with anti-human μ-, anti-human δ-, or anti-human γ-chain immunosorbent-purified rabbit antibodies. As shown in Figure 2, the depletion of cell suspensions in lymphocytes able to form rosettes with SpA-HRBC resulted in an increased number of E-RFC and a decreased number of Ig-bearing cells. The number of either IgM- or IgD-bearing lymphocytes recovered at the interface of the gradient was also significantly reduced, whereas IgG-bearing cells were no more detectable. The results of these experiments suggest that either IgG-bearing lymphocytes or a noticeable proportion of IgM- and/or IgD-bearing lymphocytes are able to form rosettes with SpA-HRBC.

Effect of depletion in IgM- and/or IgD-bearing cells. To better determine the nature of B cell subsets other than IgG-bearing lymphocytes capable of reacting with SpA-HRBC, tonsil suspensions were rosetted with a mixture of either HRBC coated with immunosorbent-purified anti-human μ-chain or

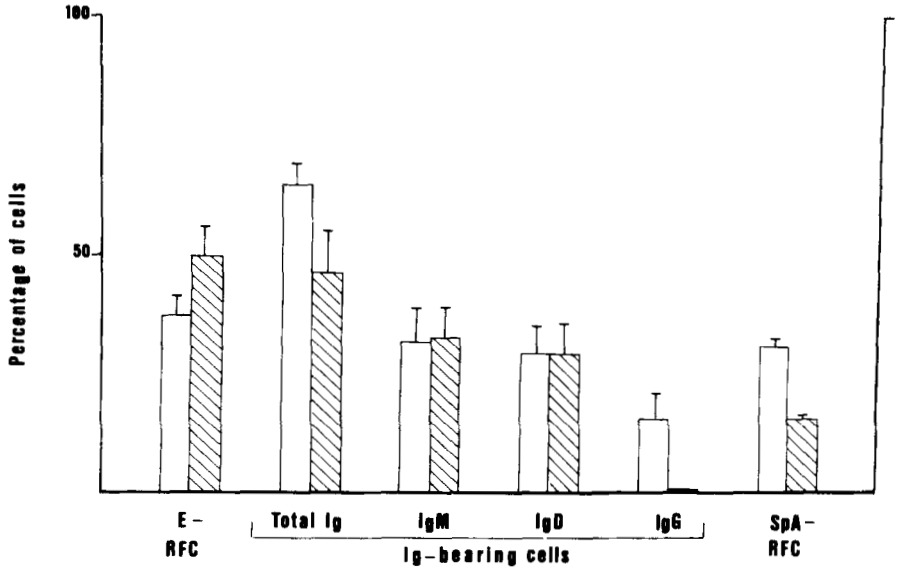

Figure 1. Effect of depletion in IgG-bearing cells on the ability of tonsil lymphocytes to form rosettes with SpA-HRBC. Tonsil cell suspensions were rosetted with HRBC coated with anti-human γ-chain immunosorbent-purified rabbit antibodies. Nonrosetted were then separated from rosetted lymphocytes by centrifugation on a Ficoll-Hypaque density gradient. The number of E-RFC and of cells forming rosettes with HRBC coated with anti-$F(ab')_2$ (Ig-bearing cells), anti-μ-chain (IgM-bearing cells), anti-δ-chain (IgD-bearing cells) rabbit antibodies or with SpA (SpA-RFC) was evaluated in unfractionated suspensions (□) and in suspensions recovered at the interface of the gradient (▨). The mean value (±S.E.) of four separate experiments is reported.

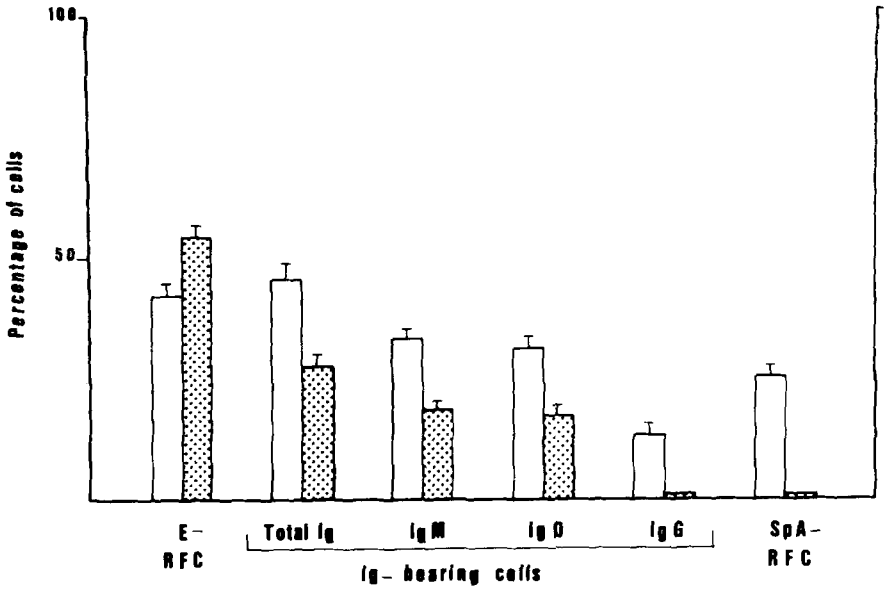

Figure 2. Effect of depletion in cells forming rosettes with SpA-HRBC. The cells forming rosettes with SpA-HRBC were removed from tonsil suspensions by centrifugation on a Ficoll-Hypaque density gradient. The number of E-RFC, of Ig-, IgM-, IgD-, IgG-bearing cells, and of cells forming rosettes with SpA-HRBC was evaluated in unfractionated suspensions (□) and in suspensions recovered at the interface of the gradient (▨). The mean value (±S.E.) of six separate experiments is reported.

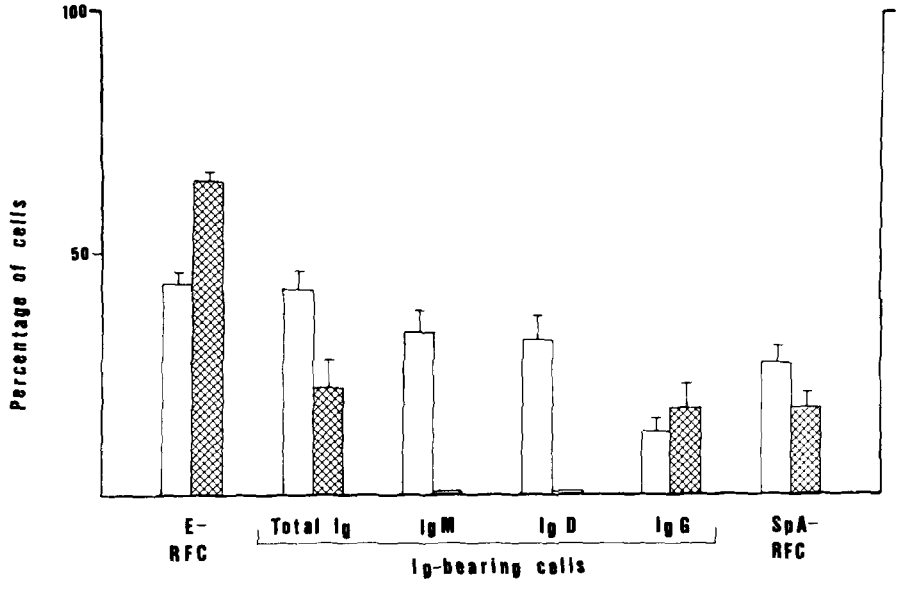

Figure 3. Effect of depletion in IgM- and/or IgD-bearing cells. Tonsil cell suspensions were rosetted with a mixture of HRBC coated with anti-human μ-chain and of HRBC coated with anti-human δ-chain rabbit antibodies. Nonrosetted lymphocytes were then separated from rosetted lymphocytes by centrifugation on a Ficoll-Hypaque density gradient. The number of E-RFC, of Ig-, IgM-, IgD-, and IgG-bearing cells, and of cells forming rosettes with SpA-HRBC was evaluated in unfractionated suspensions (□) and in suspensions recovered at the interface of the gradient (▨). The mean value (±S.E.) of four separate experiments is reported.

HRBC coated with anti-human δ-chain rabbit antibodies. Nonrosetted were then separated from rosetted lymphocytes by centrifugation on a Ficoll-Hypaque density gradient. Figure 3 summarizes the results of these experiments. The depletion in IgM- and/or IgD-bearing lymphocytes induced an increase in the number of E-RFC and a significant decrease in the number of Ig-bearing cells. On the other hand, after depletion in IgM- and/or IgD-bearing lymphocytes, the number of cells forming rosettes with SpA-HRBC was significantly reduced, and paralleled that of IgG-bearing lymphocytes. These results are consistent with those reported above, showing that SpA is capable of binding to IgG-bearing cells, but also to a remarkable subpopulation of IgM- and/or IgD-bearing lymphocytes from human tonsil.

Effect of pronase treatment on the ability of cells to form rosettes with SpA-HRBC. In order to investigate the nature of the B cell membrane components reacting with SpA-HRBC, T cell-depleted tonsil suspensions were treated with various concentrations of pronase, as reported above. The cells were then tested after the treatment to check whether the membrane components reacting with SpA had been removed. Cells were also tested for their ability to form rosettes with HRBC coated with anti-human μ-, anti-human δ-, or anti-human γ-chain antibodies before and after pronase treatment. In agreement with the results previously reported from Ferrarini *et al.* (17), the treatment of tonsil cells with pronase revealed a differential sensitivity to proteolysis of membrane IgM and IgD, IgD being removed by much lower amounts of pronase than IgM. In addition, membrane IgG showed a sensitivity to proteolysis greater than IgM, but lower than IgD, whereas the membrane components of B cells reacting with SpA-HRBC showed a sensitivity to treatment with pronase different from that of IgM, IgD, and IgG. In fact, a concentration of 50 μg/ml of pronase, which had no effect on membrane IgM or IgG, proved capable of removing IgD from more than 50% of cells and the membrane components reacting with SpA from more than 30% of lymphocytes forming rosettes with SpA-HRBC. On the other hand, after treatment with a concentration of 1.25 mg/ml of pronase, virtually no IgG and IgD could be found, whereas a noticeable proportion of cells formed rosettes with HRBC coated with anti-μ-chain antibodies, and a small but significant number of lymphocytes still showed the ability to form rosettes with SpA-HRBC (Fig. 4). In these experiments it was also found that the membrane components of B cells reacting with

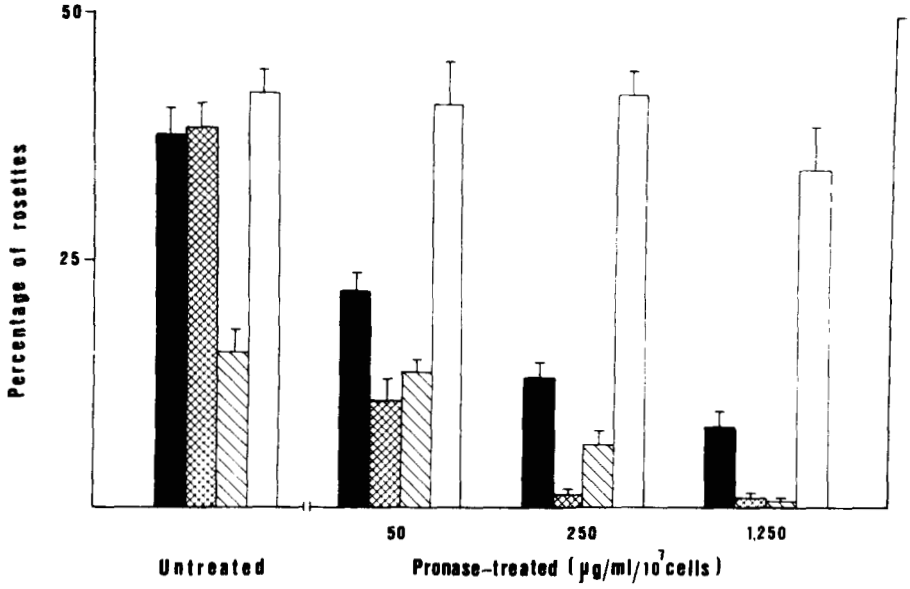

Figure 4. Sensitivity of tonsil lymphocyte membrane components responsible for the interaction with SpA-HRBC to the treatment with different concentrations of pronase. 1×10^6 cells in 0.1 ml of medium were incubated for 30 min at 37°C with 0.1 ml of medium containing the appropriate concentration of pronase. After three washings, the cells were tested for their ability to form rosettes with SpA-HRBC (■) and with HRBC coated with anti-human δ-chain (▩), anti-human γ-chain (▨), or anti-human μ chain (□) rabbit antibodies. The mean percent values (±S.E.) obtained in four separate experiments are reported.

TABLE II

Ability of T cell-depleted tonsil lymphocytes to re-express the membrane components reacting with SpA after pronase treatment[a]

Cell Suspension	% of Cells Forming Rosettes with SpA-HRBC[b]
Untreated	37.3 ± 3.0
Treated with pronase and cultured for:	
0 hr	13.4 ± 1.7
18 hr	32.6 ± 4.1

[a] Cells were treated with 250 μg/ml of pronase for 30 min at 37°C.
[b] Mean values (±S.E.) of four separate experiments.

TABLE III

Inhibition of rosettes formed by tonsil lymphocytes with SpA-HRBC by incubation with anti-human μ-chain, anti-human δ-chain or anti-human γ-chain F(ab')₂ fragments[a,b]

Inhibitor	% of Rosettes using HRBC Coated with			
	SpA	Anti-μ	Anti-δ	Anti-γ
None (PBS)	21 ± 3	34 ± 2	30 ± 2	7 ± 3
SpA[c]	<1			
F(ab')₂ anti-[d]				
μ	13 ± 2	4 ± 2	28 ± 3	8 ± 1
δ	10 ± 2	32 ± 3	<1	7 ± 2
γ	14 ± 2	33 ± 3	29 ± 2	<1
μ + δ + γ	2 ± 1	3 ± 1	<1	<1

[a] The mean values (±S.E.) of three separate experiments are reported.
[b] 1×10^6 cells in 0.1 ml of medium were incubated for 1 hr at 4°C with 0.1 ml of PBS alone or PBS containing the appropriate concentration of the inhibitor, washed twice at 4°C and tested for their ability to form rosettes with HRBC coated with SpA, anti-human μ, anti-human δ or anti-human γ-chain immunosorbent-purified rabbit antibodies.
[c] SpA was used at a concentration of 10 μg/10^6 cells.
[d] F(ab')₂ fragments, obtained by pepsin digestion from immunosorbent-purified rabbit antibodies, were used at a concentration of 10 μg/10^6 cells.

SpA were re-expressed by the great majority of lymphocytes after 18 hr in culture (Table II). These data suggest that the components of B lymphocytes reacting with SpA are not passively absorbed on the membrane and can be actively synthesized from the cells. They also provide evidence that SpA-HRBC are able to interact with membrane components of B lymphocytes showing differential sensitivity to the treatment with pronase.

TABLE IV

Inhibition of rosettes formed by tonsil lymphocytes with HRBC coated with anti-μ or anti-δ F(ab')₂ fragments by preincubation of cells with SpA[a]

Inhibitor	% of Rosettes using HRBC Coated with		
	SpA	Anti-μ F(ab')₂	anti-δ F(ab')₂
None (PBS)	25 ± 2	34 ± 6	30 ± 3
SpA (μg/10^6 cells)			
1	6 ± 2	25 ± 6	28 ± 2
10	<1	20 ± 6	20 ± 1
100	<1	18 ± 5	16 ± 2

[a] 1×10^6 cells in 0.1 ml of medium were incubated for 1 hr at 4°C with 0.1 ml of PBS alone or PBS containing the appropriate concentration of SpA, washed twice at 4°C and tested for their ability to form rosettes with HRBC coated with SpA, anti-human μ- or anti-human δ-chain F(ab')₂ fragments. The mean values (±S.E.) of four separate experiments are reported.

Inhibition of rosette formation. In order to better determine the nature of the membrane components of human B lymphocytes capable of reacting with SpA-HRBC, tonsil cell suspensions were incubated with anti-human γ-, anti-human μ-, or anti-human δ-chain F(ab')₂ fragments, prepared by pepsin digestion from immunosorbent-purified rabbit antibodies, before testing for their ability to form rosettes with SpA-HRBC. The results of these experiments are summarized in Table III. The incubation with either anti-human γ-, anti-human μ-, or anti-human δ-chain F(ab')₂ fragments induced a significant decrease in the number of rosettes formed by cells with SpA-HRBC. After incubation of cell suspensions with a mixture of anti-human γ-, anti-human μ-, and anti-human δ-chain F(ab')₂ fragments, virtually no lymphocytes forming rosettes with SpA-HRBC could be found. On the other hand, after incubation with the same concentrations of anti-human γ-chain F(ab')₂ fragments, only rosettes formed with HRBC coated with anti-human γ-chain antibodies were inhibited. The incubation with anti-human μ-chain F(ab')₂ fragments inhibited only rosettes

formed with HRBC coated with anti-human μ-chain antibodies, and the incubation with anti-human δ-chain $F(ab')_2$ fragments inhibited only rosettes formed with HRBC coated with anti-human δ-chain antibodies.

The effect of SpA preincubation on the ability of tonsil lymphocyte suspensions to bind HRBC coated with anti-μ or anti-δ $F(ab')_2$ fragments was also investigated. The incubation with SpA significantly reduced the number of rosettes formed by cells with HRBC coated with either anti-μ or anti-δ $F(ab')_2$ fragments (Table IV).

These data suggest that SpA coupled to HRBC is able to react with IgG, but also with membrane components strictly related to IgM and/or IgD on the surface of a remarkable proportion of B lymphocytes from human tonsil.

DISCUSSION

The ability of *Staphylococcus aureus* bacteria (strain Cowan I with surface-bound SpA) or SpA-coated erythrocytes to adhere to the surface of IgG-carrying cells is well known (3–5). Cell lines carrying surface-localized IgG usually formed a high percentage of rosettes with SpA-containing staphylococci or SpA-coated erythrocytes, whereas cell lines carrying surface localized IgM did not (5). After incubation with anti-IgM serum, the percentage of rosette-forming cells in IgM-carrying cell lines increased drastically (5). Thus, it was concluded that SpA labeled with fluorescein isothiocyanate, ^{125}I, or bound to erythrocytes can be used as a specific reagent to demonstrate and assay IgG antibodies bound to tissue and cell surface antigens (18–20).

The experiments reported in this paper showed that a noticeable proportion of B lymphocytes from human tonsil are able to form rosettes with SpA-HRBC without previous incubation of tonsil cells with anti-Ig sera. This could indicate that many B cells from tonsil contain on the surface molecules of the IgG class. However, the number of cells forming rosettes with HRBC coated with anti-human γ-chain antibodies was significantly lower than that of cells forming rosettes with SpA-HRBC. In addition, when tonsil cell suspensions were depleted in IgG-bearing lymphocytes, a proportion of Ig-bearing cells consistently greater than 30% still showed the ability to form rosettes with SpA-HRBC. On the other hand, when tonsil lymphocyte suspensions were depleted in cells forming rosettes with SpA-HRBC, IgG-bearing cells were no more detectable, but the number of IgM-bearing and of IgD-bearing lymphocytes was also significantly reduced. Finally, in tonsil suspensions depleted in IgM- and/or IgD-bearing cells, the number of cells forming rosettes with SpA-HRBC was significantly reduced in comparison with unfractionated populations, and paralleled that of IgG-bearing lymphocytes.

In another series of experiments, the ability of these different B cell subsets from human tonsil to proliferate *in vitro* in the presence of soluble SpA, SpA coupled to Sepharose beads, and SpA-containing staphylococci was investigated. The results of the experiments showed that the response to SpA, when presented to the cells on an insoluble matrix, is peculiar of lymphocyte subsets able to form rosettes with SpA-HRBC, and it is due to either IgG-bearing or IgM- and/or IgD-bearing lymphocyte subpopulations (21).

All these data strongly suggest that SpA attached to HRBC is able to react with IgG-bearing cells, but also with a subpopulation of IgM- and/or IgD-bearing lymphocytes. This finding may be explained in several ways. First, the binding of SpA-HRBC to IgM- and/or IgD-bearing cells is due to the presence on the membrane of cytophilically bound IgG, which cannot be revealed by rosetting with HRBC coated with anti-γ-chain antibodies, but are detectable by SpA-rosetting. Previously published studies showed very low proportions of "third" population cells and of cells equipped with receptor for the Fc fragment of IgG in tonsil suspensions (22, 23). More recently, it was shown that tonsil cells lack the "third" population, but Fc receptors are clearly demonstrable on a high proportion of tonsillar B lymphocytes by appropriate immune complex test systems (24). However, the evidence is strongly against the possibility that the binding of SpA-HRBC to IgM- and/or IgD-bearing cells is due to an interaction with IgG adhering to these cells via Fc receptors. First, the number of cells forming rosettes with SpA-HRBC found in cell suspensions exposed before testing to procedures capable of removing cytophilically bound IgG, like overnight incubation in medium containing 10% FCS (25) or repeated washings at 37°C (26), was not significantly different from that of untreated or not preincubated suspensions. In addition, the experiments in which the cells were treated with pronase, and membrane immunoglobulin re-expression *in vitro* was determined, clearly showed that the components of B lymphocytes reacting with SpA-HRBC are not passively absorbed on the membrane, but are actively synthesized by cells.

A second possibility is that IgM- and/or IgD-bearing lymphocytes contain a subset of cells that also express autochthonous IgG on the membrane, but γ-chains are much less than μ- or δ-chains. Thus, it is possible that for the detection of γ-chains, only the rosette test with SpA-HRBC is operating well within its limit of sensitivity. Another possibility is that rosettes formed with SpA-HRBC by IgM- and/or IgD-bearing cells are due to an interaction between SpA and membrane immunoglobulin other than IgG, like IgM and/or IgD. It has been shown that SpA is able to interact with the Fc region of some IgM monoclonal immunoglobulins (27) and IgM synthesized *in vitro* by a human lymphoblastoid cell line (28). On the other hand, in immunoprecipitation studies it was found that a high proportion of human lymphocyte surface immunoglobulins of different classes bound directly to SpA-containing staphylococci (29).

The experiments here reported, in which cells were treated with different concentrations of pronase, suggest that SpA can bind to membrane components of B lymphocytes showing different sensitivity to proteolytic enzymes. On the other hand, the present studies also indicate that the binding of SpA-HRBC to Ig-bearing human lymphocytes can be partially inhibited by preincubation of the cells with anti-human γ-chain, but also with anti-human μ-chain or anti-human δ-chain $F(ab')_2$ fragments. After incubation of lymphocytes with a mixture of anti-human γ-chain, anti-human μ-chain, and anti-human δ-chain $F(ab')_2$ fragments, virtually no cells forming rosettes with SpA-HRBC could be found. In addition, SpA preincubation was able to reduce significantly the binding of cells to HRBC coated with anti-μ or anti-δ $F(ab')_2$ fragments. These data, obviously, do not prove that SpA binds directly to either IgM or IgD. In fact, it cannot be excluded that the mechanism for inhibition could result from steric hindrance of an adjacent receptor. However, the results reported in the present paper clearly suggest that SpA interacts with IgG, but also with membrane components strictly related to IgM and/or IgD present on the surface of a remarkable proportion of human lymphoid cells.

Of the central importance to the validity of this conclusion is the nature of the assays employed for detecting immunoglobulin on the lymphocyte membrane. It has been shown that

rosetting with chromic chloride is more sensitive than immunofluorescence (30, 31). On the other hand, the results of inhibition experiments clearly indicate that monospecific reagents were used by us through the present study. The possible contribution of surface IgA- and IgE-bearing cells present in tonsil tissue to the formation of rosettes with SpA-HRBC was not evaluated in this series of experiments. Since SpA is known to react also with some monoclonal IgA immunoglobulins (32), colostral IgA (33), and serum IgE (34), further studies are expected in order to elucidate this point.

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