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Protein A of *Staphylococcus aureus* is mitogenic for IgG-bearing, but also for a subpopulation of IgM- and/or IgD-bearing human lymphocytes

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Summary. The nature of lymphocyte subsets activated by soluble and insoluble protein A (SpA) was investigated by testing the ability of human tonsil populations to form rosettes with human red blood cells coated with SpA (SpA-HRBC) and to respond *in vitro* to SpA, SpA coupled to Sepharose beads (SpA-Seph) and *Staphylococcus aureus* strain Cowan I (StaCw).

Purified human T cells, which were unable to form rosettes with SpA-HRBC, were not activated by SpA-Seph or StaCw, whereas B-cell enriched suspensions, where the number of lymphocytes forming rosettes with SpA-HRBC was significantly increased in comparison with that found in unfractionated populations, showed DNA synthesis equal to or greater than unseparated lymphocytes. In contrast, soluble SpA was unable to activate highly purified B lymphocytes in 3 day cultures and induced higher DNA synthesis in unseparated than in purified human T cells. Tonsil cell suspensions depleted in cells forming rosettes with SpA-HRBC synthesized significantly less DNA in the presence of SpA-Seph and lost the ability to respond to StaCw. The depletion in either IgG-bearing or IgM- and/or IgD-bearing cells induced a reduction in the response of lymphocytes to SpA-Seph and StaCw. Depletion in IgM- and/or IgD-bearing cells induced a more marked decrease in the response to StaCw than depletion in IgG-bearing lymphocytes, while the decrease of the response to SpA-Seph, induced by depletion in IgM- and/or IgD-bearing cells, was lower than that induced by depletion in IgG-bearing lymphocytes. In contrast, soluble SpA-induced proliferation was not significantly affected by depletion of cells forming rosettes with SpA-HRBC or of IgG-bearing or IgM- and/or IgD-bearing lymphocytes. These results suggest that the mitogenicity of SpA-Seph and StaCw is due to a selective binding of insoluble SpA to components present on the membrane of either IgG-bearing or IgM- and/or IgD-bearing lymphocytes. They also indicate that the potentiation of T-cell response to soluble SpA, induced by the presence in culture of non-T cells, is not due to B lymphocytes which are able to form rosettes with SpA-HRBC and to respond to SpA itself when it is presented to the cells on an insoluble matrix.

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INTRODUCTION

Protein A of *Staphylococcus aureus* (SpA) has been shown to bind specifically to the Fc region of IgG immunoglobulins from several species (Forsgren & Sjöquist, 1966; Sjöquist, Forsgren, Gustafson & Stålenheim, 1967). More recently, it was found that *S. aureus* bacteria strain Cowan I (StaCw), containing SpA, are an efficient mitogen for human B lympho-

cytes, with no detectable activity for T lymphocytes (Forsgren, Svedjelund & Wigzell, 1976; Romagnani, Amadori, Giudizi, Biagiotti, Maggi & Ricci, 1978) and are also capable of inducing polyclonal antibody secretion in human peripheral blood lymphocytes (Ringdén, Rynnel-Dagöö, Waterfield, Möller & Möller, 1977; Rynnel-Dagöö, Ringdén, Alfredsson & Möller, 1978).

The exact nature of B-cell subsets activated by StaCw and the mechanism by which these bacteria exert their stimulating activity are unknown. It was suggested that the mitogenicity of StaCw can be due to a selective binding of SpA to IgG molecules present on the surface of B lymphocytes (Forsgren *et al.*, 1976). However, it is known that peripheral blood B cells with intrinsic membrane IgG are usually less than 1% of total peripheral blood lymphocytes (Winchester, Fu, Hoffman & Kunkel, 1975). In addition, the cells which passively adsorb IgG by their receptor for the Fc fragment seem unable to proliferate *in vitro* in the presence of StaCw (Möller & Landwall, 1977; Romagnani *et al.*, 1978). Finally, soluble SpA was found to be an efficient mitogen also for human T lymphocytes (Sakane & Green, 1978; Rynnel-Dagöö *et al.*, 1978; Romagnani *et al.*, 1978). More recently, we were able to show that SpA coupled to human erythrocytes is capable of binding to IgG-bearing cells, but also to a subpopulation of IgM- and/or IgD-bearing lymphocytes from human tonsil (Romagnani, Giudizi, Almerigogna & Ricci, submitted for publication).

In the present paper, we report experiments undertaken to characterize human lymphocyte subsets activated *in vitro* by SpA and StaCw.

MATERIALS AND METHODS

Staphylococci

S. aureus strains Cowan I (with a high content of protein A) (StaCw) and Wood 46 (with a low content of protein A) (StaWo) originally obtained from the National Collection of Type Cultures (London) were used. The bacteria were killed by incubation in 0·5% formaldehyde for 3 h at room temperature, heat-treated at 80° for 3 min, washed and finally stored in small aliquots at −80°.

Staphylococcal protein A

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

Anti-immunoglobulin (Ig) antisera

All anti-human Ig antisera were prepared in rabbits and purified with immunosorbents. For immunization, 2 mg of protein were emulsified in Freund's complete adjuvant (FCA; Difco, Detroit) and injected intramuscularly and into the footpads. Rabbits were boosted 15 days later with intramuscular injections of 2 mg of protein emulsified in FCA and 30 days later with intramuscular injections of 2 mg of protein emulsified in incomplete Freund's adjuvant (Difco, Detroit). Antiserum specific for human IgM was prepared by injecting rabbits with IgM obtained from pooled sera of macroglobulinaemia patients by euglobulin precipitation followed 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 (Rowe, Dolder & Welscher, 1969). 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 purified 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 (Pharmacia, Uppsala) to remove undigested IgG (Hjelm, Hjelm & Sjoquist, 1972). 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-coupled Sepharose CL-4B to remove contaminating IgG (Hjelm *et al.*, 1972). Human IgG, IgM, IgA, IgD and $F(ab')_2$ were coupled on CNBr-activated Sepharose 4B (Axén & Ernback, 1971). For the purification of anti-IgM (anti-μ) antibody, the anti-IgM serum was passed through an IgG, an IgA, an IgD and an $F(ab')_2$ 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 OD units at 280 nm. 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, and IgA and an $F(ab')_2$ immunosorbent column.

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 an $F(ab')_2$, an IgM, an IgD and an IgA column. The effluent was then passed over an IgG immunosorbent column and the antibody isolated by acidic elution. Purified anti-$F(ab')_2$ antibodies were prepared by elution of the anti-$F(ab')_2$ serum through the $F(ab')_2$ immunosorbent column followed by acidic elution, as described above.

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 described by Ceska & Lundkvist (1972). IgM, IgD or IgG molecules were allowed to bind to antibody-coated discs. ^{125}I-radio-iodinated, immunosorbent-purified antibodies were then allowed to react with the bound molecules. Anti-μ antiserum was found capable of reacting with IgM only, anti-δ antiserum with IgD only and anti-γ antiserum with IgG 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 & Fudenberg (1967). Briefly, one volume of washed and packed HRBC and one volume of immunosorbent-purified anti-human μ chain, anti-human δ chain, anti-human γ chain, anti-human $F(ab')_2$ antibody (1 mg/ml) or of SpA (1 mg/ml) in 0·15 M NaCl were mixed. Then, one volume of $CrCl_3.6H_2O$ (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 of tonsil lymphocytes

Palatin tonsils from unselected patients were collected under sterile conditions in cold TC 199 medium (Difco, Detroit), supplemented with 1000 units/ml of penicillin G, 500 μg/ml of streptomycin, 100 μg/ml of kanamycin and 1 μg/ml of gentamycin. Within 30 min after tonsillectomy, the tonsil was gently sliced in an ice bath and the tissue 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. Phagocytic cells were removed by magnetism. The cells recovered were washed three times and used either directly or after various other purification procedures.

Rosette assay with anti-human Ig- or SpA-coated HRBC and Ficoll–Hypaque separation of rosetted lymphocytes

One volume of the lymphocyte suspension (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°. The pellet was resuspended by pipetting, stained with toluidine blue and the percentage of rosettes was determined by microscopic examination of at least 300 cells. In order to separate non-rosetted from rosetted lymphocytes, 1 ml of the rosetted mixture was layered on 2 ml Ficoll–Hypaque and centrifuged at 400 ***g*** for 30 min at 4°. After centrifugation, the cells recovered at the interface were collected (Strelkauskas, Teodorescu & Dray, 1975).

Preparation of purified T lymphocytes

To obtain pure T lymphocytes, tonsil populations were rosetted with neuraminidase-treated sheep red blood cells (SRBC), as detailed elsewhere (Romagnani, Maggi, Amadori, Giudizi & Ricci, 1977; Romagnani *et al.*, 1978). 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 and one volume of the lymphocyte suspension incubated with HRBC coated with anti-human $F(ab')_2$ immunosorbent-purified rabbit antibodies. The mixture was centrifuged on a Ficoll–Hypaque density gradient and the cells of the interface were recovered. This double separation procedure gave suspensions virtually free from non-T cells.

Preparation and fractionation of non-T cells

Suspensions virtually free from T lymphocytes were obtained from tonsil populations by a double E-rosetting procedure, previously reported in detail (Romagnani *et al.*, 1977; Romagnani *et al.*, 1978). Briefly, the mononuclear cells recovered from the Ficoll–Hypa-

que monolayer were E-rosetted with neuraminidase-treated SRBC. E-RFC were separated from non E-RFC on a Ficoll–Hypaque density gradient. The cells recovered at the interface of the gradient were re-rosetted with neuraminidase-treated SRBC and centrifuged on another density gradient. The suspensions recovered at the interface after this double-step procedure consistently contained less than 1% E-RFC. We will refer to this preparation as non-T cells.

IgM- and/or IgD-bearing cells, IgG-bearing cells or cells capable of forming rosettes with SpA-HRBC were removed by rosetting cell suspensions with HRBC coated with anti-human μ and anti-human δ chain, anti-human γ chain or SpA, respectively. Non-rosetted were then separated from rosetted lymphocytes by centrifugation on a Ficoll–Hypaque density gradient, as described above.

Cell cultures

Whole lymphocytes obtained from the Ficoll–Hypaque monolayer, as well as cell suspensions recovered after various purification procedures, were resuspended at 2×10^6 cells/ml in complete culture medium. This complete culture medium used for all these studies consisted of TC 199 supplemented with 100 units penicillin/ml, 100 μg streptomycin/ml, 50 μg kanamycin/ml, 2 mM L-glutamine and 20% heat-inactivated foetal calf serum (FCS; Grand Island Biological Co., Grand Island, N.Y.). Lymphocyte cultures were established in microtitre V plates (Sterilin, Teddington, Middlesex) in quadruplicate and consisted of 0·1 ml of complete culture medium containing 2×10^5 cells and 0·1 ml of medium alone or medium containing the appropriate concentration of the stimulant. All cultures were incubated for 72 h at 37° in a 5% CO_2/95% air humidified environment. Sixteen hours before harvesting, 0·5 μCi [methyl-^{3}H]-thymidine (^{3}H-TdR; 24 Ci/mmol; The Radiochemical Centre, Amersham) was added to each culture well. At the end of the incubation period, the cells were processed on a harvesting machine (Skatron, Lierbyen, Norway) and incorporation of ^{3}H-TdR was measured in a Packard liquid scintillation counter.

Stimulants

StaCw were used at concentrations ranging between 1 and 500×10^6 bacteria/ml; soluble SpA at concentrations ranging between 0·1 and 250 μg/ml; SpA-Seph at concentrations ranging between 0·05 and 2·5 mg/ml. PHA M (Difco, Detroit) was dissolved with 5 ml of medium and used at concentrations ranging between 0·1 and 100 μl/ml.

RESULTS

Different mitogenic activity of SpA, SpA-Seph and StaCw on unseparated, purified T, and non-T tonsil lymphocytes

The capacity of unfractionated, T and non-T tonsil cells to respond to stimulation with soluble SpA, SpA-Seph and StaCw for 3 days is presented in the Fig. 1. The magnitude of ^{3}H-TdR incorporated by non-T cells in response to StaCw and SpA-Seph was equal to or slightly lower than the response of unseparated cells, whereas purified T lymphocytes were not activated by StaCw and gave a small response in the presence of SpA-Seph. In contrast, soluble SpA displayed a high degree of mitogenic activity on unseparated populations, but it was unable to activate T-cell depleted suspensions. On the other hand, the response to soluble SpA of purified T lymphocytes was consistently lower than the response of unseparated populations. The PHA response of these unseparated and separated populations is also illustrated in Fig. 1. Either unfractionated lymphocytes or separated T cells displayed a high degree of response when incu-

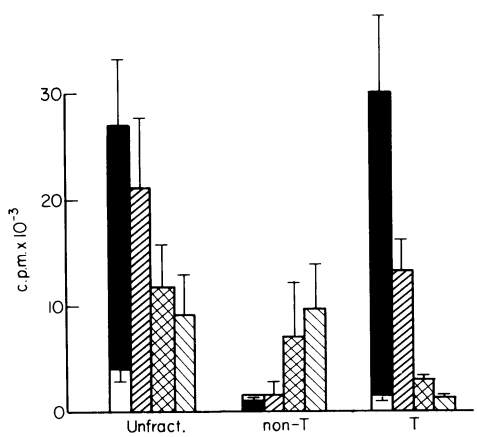

Figure 1. Incorporation of ^{3}H-TdR by unfractionated, purified T and non-T lymphocytes from human tonsil after incubation for 3 days in culture with PHA, 10 μg/ml (solid columns), SpA, 100 μg/ml (narrow-hatched columns), SpA-Seph, 500 μg/ml (cross-hatched columns), StaCw, 25×10^6/ml (wide-hatched columns) or without mitogen (open columns). Results are expressed as c.p.m. and represent the mean value ($\pm$SE) of seven separate experiments.

bated with PHA. In contrast, the non-T lymphocytes were not stimulated by this mitogen. Thus, the results of these experiments provide further evidence that SpA in a soluble form has a PHA-like mitogenic activity on unseparated cells, but it is a weaker mitogen than PHA for purified T cells. In contrast, insoluble SpA and StaCw exhibit mitogenic activity only for non-T lymphocytes.

Effect of depletion in cells forming rosettes with SpA-HRBC on the response of lymphocytes to SpA, SpA-Seph and StaCw

In order to investigate the nature of cell subsets responsible for the proliferation induced by SpA-Seph and StaCw, tonsil lymphocytes were rosetted with SpA-HRBC. Non-rosetted were then separated from rosetted lymphocytes by centrifugation of the cell mixture on a Ficoll–Hypaque density gradient and cultured *in vitro* for 3 days in the presence of soluble SpA, SpA-Seph and StaCw. Figure 2 shows that cells forming rosettes with SpA-HRBC are B lymphocytes. In fact, purified T lymphocytes did not form rosettes with SpA-HRBC, whereas in T-cell depleted suspensions the proportion of either Ig-bearing cells or lymphocytes reacting with SpA-HRBC was significantly increased in comparison with that of unseparated populations.

As shown in Table 1, the depletion of tonsil cell

Table 1. Effect of depletion in cells forming rosettes with SpA-HRBC on the percentage of E-RFC, and of cells forming rosettes with HRBC coated with anti-F(ab′)$_2$, anti-μ chain and anti-γ chain immunosorbent-purified rabbit antibodies*

Cell population	% of E rosettes	% of rosettes using HRBC coated with: anti-F(ab′)$_2$	anti-μ	anti-γ	SpA
Unfractionated	44 ± 1	39 ± 4	32 ± 2	11 ± 4	21 ± 4
Depleted in cells forming rosettes with SpA-HRBC	56 ± 2	24 ± 4	18 ± 2	< 1	< 1

* Results are expressed as mean percentage (± SE) of six separate experiments

suspensions in lymphocytes able to form rosettes with SpA-HRBC resulted in an increased number of E-RFC and in a decreased number of Ig-bearing cells. The number of IgM-bearing lymphocytes recovered at the interface of the gradient was also significantly reduced, while IgG-bearing cells were no longer detectable.

The ability of tonsil suspensions, depleted in cells forming rosettes with SpA-HRBC, to respond to stimulation with the mitogens mentioned above is presented in Fig. 3. The depletion in cells forming rosettes

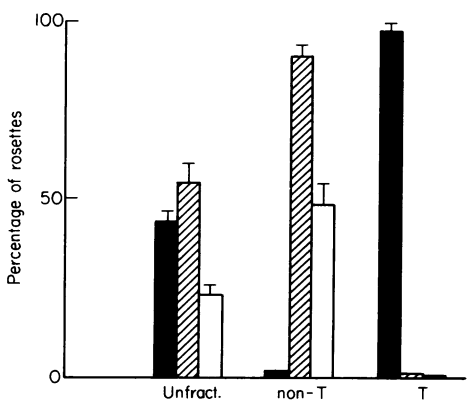

Figure 2. Ability of unfractionated, purified T and non-T lymphocytes from human tonsil to form rosettes with SpA-HRBC. The mean percent values (± SE) of E-RFC (solid columns), cells forming rosettes with HRBC coated with anti-human F(ab′)$_2$ rabbit antibodies (hatched columns) and cells forming rosettes with SpA-HRBC (open columns) found in six separate experiments are reported.

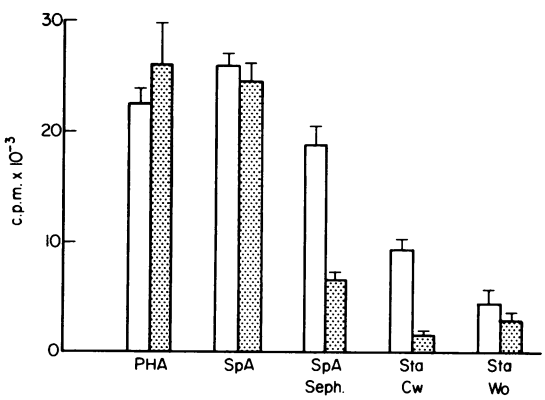

Figure 3. Incorporation of [^{3}H]-TdR by unfractionated tonsil cell suspension (open columns) and cell suspensions depleted in cells forming rosettes with SpA-HRBC (hatched columns) after stimulation for 3 days in culture with PHA (10 μl/ml), SpA (100 μg/ml), SpA-Seph (500 μg/ml), StaCw (25×10^6/ml) and StaWo (25×10^6/ml). Results are expressed in c.p.m. in stimulated cultures minus c.p.m. in unstimulated cultures and represent the mean value (± SE) of four separate experiments.

Table 2. Effect of depletion in IgG-bearing cells on the ability of lymphocyte suspensions from human tonsil to form rosettes with SpA-HRBC*

Cell population	% of E rosettes	% of rosettes using HRBC coated with anti-F(ab')$_2$	anti-μ	anti-δ	anti-γ	SpA
Unfractionated	37±2	54±4	32±3	29±3	12±3	30±1
Depleted in IgG-bearing cells	47±3	44±4	33±3	30±3	<1	16±1

* Results are expressed as mean percentage (±SE) of four separate experiments.

with SpA-HRBC induced a marked decrease in the response to SpA-Seph and virtually abolished the response to StaCw, whereas the responses to soluble SpA and PHA were unchanged. On the other hand, the weak mitogenic response to StaWo, a strain which contains a very small amount of SpA, was not significantly decreased.

Effect of depletion in IgG-bearing cells on the response of lymphocytes to SpA-Seph and StaCw

In order to establish whether or not B lymphocytes capable of reacting with insoluble SpA were the cells equipped with membrane IgG, lymphocytes were rosetted with HRBC coated with immunosorbent-purified anti-human γ chain rabbit antibodies. Non-rosetted were then separated from rosetted lymphocytes by centrifugation on a Ficoll–Hypaque density gradient. As shown in Table 2, the depletion in cells forming rosettes with HRBC coated with anti-human γ chain rabbit 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 of 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 significantly decreased. However, in the interface population, 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. The capacity of tonsil suspensions depleted in IgG-bearing cells to respond to stimulation with soluble and insoluble SpA and with StaCw is presented in the Fig. 4. The depletion in IgG-bearing cells reduced the response of lymphocytes to SpA-Seph and, to some extent, to StaCw. On the other hand, the responses to soluble SpA and PHA of lymphocyte suspensions depleted in IgG-bearing cells were equal to or greater than those of unfractionated populations.

Effect of depletion in IgM- and/or IgD-bearing cells on the response of lymphocytes to SpA-Seph and StaCw

To determine better the nature of B-cell subsets capable of reacting with StaCw, lymphocytes were rosetted with a mixture of either HRBC coated with immunosorbent-purified anti-human μ chain or HRBC

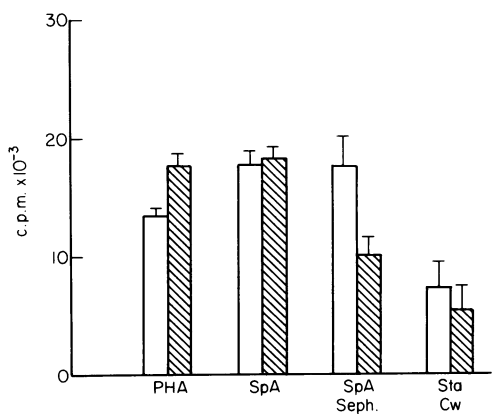

Figure 4. Incorporation of ^{3}H-TdR by unfractionated tonsil cell suspension (open columns) and cell suspensions depleted in cells forming rosettes with HRBC coated with anti-human γ chain rabbit antibodies (hatched columns) after stimulation for 3 days in culture with PHA (10 μg/ml), SpA (100 μg/ml), SpA-Seph (500 μg/ml) and StaCw (25×10^6/ml). Results are expressed in c.p.m. in stimulated cultures minus c.p.m. in unstimulated cultures and represent the mean value (±SE) of four separate experiments.

Table 3. Effect of depletion in IgM- and/or IgD-bearing cells on the ability of lymphocyte suspensions from human tonsil to form rosettes with SpA-HRBC*

Cell population	% of E rosettes	% of rosettes using HRBC coated with anti-F(ab')$_2$	anti-μ	anti-δ	anti-γ	SpA
Unfractionated	43±2	42±4	34±5	33±5	13±3	27±3
Depleted in IgM- and/or IgD-bearing cells	65±2	22±6	<1	<1	17±5	17±3

* Results are expressed as mean percentage (±SE) of four separate experiments.

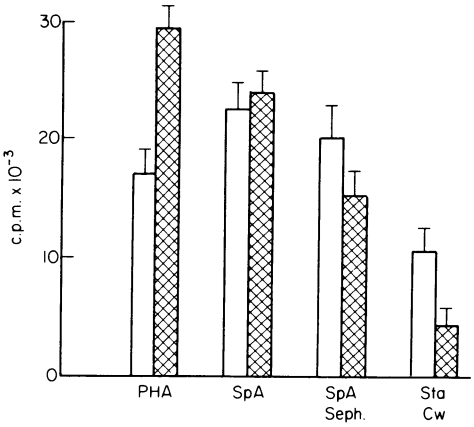

Figure 5. Incorporation of ^{3}H-TdR by unfractionated tonsil cell suspensions (open columns) and cell suspensions depleted in IgM- and/or IgD-bearing cells (hatched columns) after stimulation for 3 days in culture with PHA (10 μl/ml), SpA (100 μg/ml), SpA-Seph (500 μg/ml) and StaCw (25×10^6/ml). Results are expressed in c.p.m. in stimulated cultures minus c.p.m. in unstimulated cultures and represent the mean value (±SE) of four separate experiments.

coated with anti-human δ chain rabbit antibodies. Non-rosetted were then separated from rosetted lymphocytes by centrifugation on a Ficoll–Hypaque density gradient. Table 3 summarizes the results of these experiments. The depletion in cells bearing IgM and/or IgD on the membrane induced an increase in the number of E-RFC and a decrease in the number of Ig-bearing lymphocytes. On the other hand, after depletion in IgM- and/or IgD-bearing cells, the number of lymphocytes forming rosettes with SpA-HRBC was significantly reduced and paralleled that of IgG-bearing lymphocytes.

The capacity of suspensions depleted in IgM- and/or IgD-bearing cells to respond to stimulation with StaCw and SpA-Seph is presented in the Fig. 5. The depletion in IgM- and/or IgD-bearing cells significantly reduced the response to SpA-Seph and still more the response to StaCw, whereas the responses to soluble SpA and PHA were equal to or greater than those of unseparated populations.

DISCUSSION

We have recently reported (Romagnani *et al.*, 1978) and we can confirm now in another series of experiments that soluble and insoluble SpA have a different mitogenic activity for human lymphocytes. Soluble SpA induces higher DNA synthesis in unseparated lymphocytes than in purified human T cells, whereas T-cell depleted suspensions from human tonsil do not show a significant proliferation when cultured for 3 days in the presence of soluble SpA. Similar results have been also reported by Sakane & Green (1978) and by Ringdén & Rynnel-Dagöö (1978), who found that the best stimulation by soluble SpA is seen in unseparated lymphocytes or mixtures of T and B cells than in purified T cells. In contrast, a significant response of purified B lymphocytes to soluble SpA was reported by the same workers, but peak stimulation was found at 5 days (Sakane & Green, 1978) or 7 days (Ringdén & Rynnel-Dagöö, 1978) of culture.

With regard to the activity of SpA attached to Sepharose beads and of SpA-containing Staphylococci, our results are in agreement with those of Forsgren *et al.* (1976), showing that these mitogens are unable to activate purified human T cells and give high

DNA synthesis in either unseparated or B-cell enriched lymphocyte suspensions. In the previous paper mentioned above, we also demonstrated that the response of highly purified human B cells to SpA-containing Staphylococci is a T-cell independent phenomenon (Romagnani *et al.*, 1978). In addition, other workers found that StaCw, as well as SpA in soluble form or coupled to Sepharose beads, are capable of inducing immunoglobulin synthesis and secretion in human splenic and peripheral blood lymphocytes (Möller & Landwall, 1977; Ringdén *et al.*, 1977; Ringdén & Rynnel-Dagöö, 1978; Rynnel-Dagöö *et al.*, 1978).

All these findings raise several questions. The first is whether the interaction of StaCw with human B lymphocytes is due to the SpA present on the bacterium surface or there are other substances than protein A on *Staphylococcus* bacteria responsible for the activation of human B cells. The experiments reported in this paper showed that a noticeable proportion of human B lymphocytes from tonsil are able to form rosettes with SpA-HRBC. In addition, it was found that lymphocytes capable of forming rosettes with SpA-HRBC are the B-cell subsets responsible for the proliferation induced by StaCw. In fact, tonsil cell suspensions depleted in lymphocytes forming rosettes with SpA-HRBC, presented a significantly lower DNA synthesis in the presence of SpA-Seph and virtually lost the ability to respond to StaCw. In contrast, the proliferation induced by soluble SpA and PHA, as well as the weak mitogenic response to StaWo, were not significantly affected by the depletion in cells forming rosettes with SpA-HRBC. These data provide evidence that SpA present on the bacterial surface is responsible for cell activation detected as increase in DNA synthesis. However, there are probably other substances on the surface of *Staphylococcus* strains which are responsible for polyclonal antibody secretion in human lymphocytes. In fact, it is well known that preparations of *S. aureus* strain Wood 46 (with low content in SpA) or NG 70 (a SpA-deficient mutant), which give low or no increase in DNA synthesis, are able to induce polyclonal antibody secretion in human lymphocytes (Ringdén *et al.*, 1977; Rynnel-Dagöö *et al.*, 1978; Banck & Forsgren, 1978).

The results reported in the present paper also indicate that the response of T cells to soluble SpA is not influenced by the presence in culture of B lymphocytes able to form rosettes with SpA-HRBC and to respond to SpA itself when presented to the cells on an insoluble matrix. Thus, it is clear that other non-T cells are responsible for the potentiation of the T-cell response which is seen when unseparated populations or mixtures of T and non-T cells are challenged with soluble SpA.

Another important question is the nature of B-cell subsets capable of forming rosettes with SpA-HRBC and to proliferate *in vitro* in the presence of insoluble SpA. We have demonstrated that the depletion in either IgG-bearing or IgM- and/or IgD-bearing cells induced a decrease in the response of lymphocytes to SpA-Seph and StaCw, while the responses to soluble SpA and PHA were equal to or greater than those of unseparated populations. The depletion in IgM- and/or IgD-bearing cells induced a greater decrease in the response to StaCw than the depletion in IgG-bearing lymphocytes. In contrast, the response to SpA-Seph was less markedly decreased by the depletion in IgM- and/or IgD-bearing cells than by depletion in IgG-bearing lymphocytes.

These data are partly consistent with previously reported results showing that both SpA and StaCw are able to react with IgG-bearing lymphocytes, probably by interaction between SpA and the Fc region of surface IgG immunoglobulin (Ghetie, Fabricius, Nilsson & Sjöquist, 1974; Ghetie, Stålenheim & Sjöquist, 1975). However, in contrast to the findings of these workers, the present data clearly show that SpA and SpA-containing Staphylococci are also capable of binding to a remarkable subpopulation of cells apparently lacking membrane IgG and carrying IgM and/or IgD. They also suggest that cells responding to SpA-Seph are predominantly IgG-bearing cells, while lymphocytes which proliferate in the presence of StaCw are mainly IgM- and/or IgD-bearing cells.

In another paper, we were able to show that the membrane components of most lymphocytes reacting with SpA-HRBC are not passively absorbed on the cell surface but are actively synthesized following treatment of the cells with pronase. In addition, it was found that the membrane components of some IgM- and/or IgD-bearing lymphocytes reacting with SpA-HRBC are sensitive, like membrane IgD, to the treatment with low concentrations of pronase. In contrast, SpA-reacting membrane components of another subset of IgM- and/or IgD-bearing cells are resistant, like membrane IgM, to the treatment with higher pronase concentrations. Finally, it was also shown that the interaction with SpA-HRBC could be significantly inhibited by the incubation of cells with anti-γ chain, but also with anti-μ or anti-δ chain $F(ab')_2$ fragments. After incubation of lymphocytes with a

mixture of anti-γ, anti-μ and anti-δ chain F(ab')$_2$ fragments, virtually no cells forming rosettes with SpA-HRBC could be found (Romagnani *et al.*, submitted for publication).

These findings, together with the data reported in the present paper, suggest that the mitogenicity of SpA-Seph and StaCw may be due to the binding of insoluble SpA to IgG, but also to IgM and/or IgD (or membrane components strictly related to these immunoglobulins) on the surface of a high proportion of human B lymphocytes. In addition, the results reported here also show that SpA attached to Sepharose beads is more mitogenic for IgG-bearing cells, while SpA presented to the cells on the bacterial surface is more mitogenic for IgM- and/or IgD-bearing lymphocytes. The functional significance of this difference is unknown. The mechanism by which soluble SpA is able to activate T cells which do not seem capable of reacting with SpA attached to erythrocytes or present on the bacterial surface, also remains to be determined.

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