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Different mitogenic activity of soluble and insoluble staphylococcal protein A (SPA)

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Summary. The response to SPA and Staphylococcus strain Cowan I (StaCw) of highly purified populations of peripheral blood and tonsil human lymphocytes was investigated.

Purified T lymphocytes isolated from peripheral blood by E-rosetting were unable to respond *in vitro* to StaCw and showed a poorly significant response to soluble SPA. On the contrary B-cell-enriched suspensions of either peripheral blood or tonsil responded well to StaCw.

Either EA-RFC or EA-RFC-depleted suspensions showed a significant response to soluble SPA, but only EA-RFC-depleted suspensions were activated by StaCw.

Highly purified B-cell populations from tonsils did not show any proliferative response in the presence of soluble SPA. The addition to highly purified B-cell suspensions from human tonsils of increasing concentrations of autologous T lymphocytes did not induce any increase of thymidine uptake in the presence of StaCw. However, it was able to restore a marked proliferative response of the B-cell cultures to soluble SPA, even though mitomycin-treated T lymphocytes were added.

The low response of highly purified peripheral blood T lymphocytes to soluble SPA could be potentiated by the addition of autologous mitomycin-treated B cells, whereas the unresponsiveness of purified T lymphocytes to StaCw was not affected.

Mitogenic activity of SPA coupled to Sepharose beads was different from that of soluble SPA and paralleled that of StaCw.

These data strongly suggest that insoluble SPA is a T-cell-independent B-cell mitogen in man, whereas soluble SPA, like PWM, exerts its activity on B cells only in the presence of T cells.

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INTRODUCTION

Staphylococcal protein A (SPA), isolated from the cell walls of Staphylococcus strain Cowan I (StaCw), has been demonstrated to possess precipitating activity for IgG of several mammalian species, including man. This activity is based on the affinity of SPA for the Fc portion of IgG (Forsgren & Sjöquist, 1966). StaCw or SPA labelled with fluorescein isothiocyanate or ^{125}I have been used to identify B cells in the mouse and man (Ghetie, Fabricius, Nilsson & Sjöquist, 1974; Ghetie, Stalenheim & Sjöquist, 1975; Ades, Phillips, Shore, Gordon, Lavia, Black & Reimer, 1976; Ranki, Tötterman & Häyry, 1976), and lymphocyte separation has been achieved by SPA-coated erythrocytes (Ghetie *et al.*, 1975). More recently.

it was shown that SPA is also able to interact with the Fc fragment of some IgM and IgA monoclonal immunoglobulins (Lind, Harboe & Følling, 1975; Saltvedt & Harboe, 1976).

Until now, conflicting results have been reported with regard to the capacity of SPA to activate human peripheral blood lymphocytes. Rodey, Davis & Quie (1972) showed that SPA possesses a strong mitogenic activity on human cells and suggested that this activity is related to a proliferation of antigen-specific T lymphocytes. On the other hand, Williams & Kronvall (1972) were unable to obtain significant stimulation by SPA applied over a wide range of concentration using as cells to be stimulated human or rabbit lymphocytes. More recently, it has been reported that SPA is a highly efficient mitogen for human peripheral blood B lymphocytes, when presented to the cells on an insoluble matrix, such as the SPA-containing bacteria or SPA covalently attached to Sephadex or Sepharose beads (Forsgren, Svedjelund & Wigzell, 1976).

In this paper the response of highly purified populations of human T and B cells from peripheral blood and tonsils to soluble SPA and SPA attached to bacteria or Sepharose beads was investigated. An attempt was also made to identify the nature of the proliferating cells and to study the possible interactions among different subpopulations in the response to soluble and insoluble SPA.

MATERIALS AND METHODS

Staphylococci

S. aureus strain Cowan I (StaCw) 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 at −80°.

Staphylococcal protein A (SPA)

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

Labelling of StaCw and SPA

Labelling of StaCw by fluorescein isothiocyanate (Sigma) was carried out according to the method described by Ghetie *et al.* (1974).

Soluble SPA was fluoresceinated by the method reported by Biberfeld, Ghetie & Sjöquist (1975).

Fluorescent staining

For the detection of SPA-labelled cells, 0·1 ml of medium containing 1×10^6 lymphocytes were incubated for 30 min at 4° with 0·1 ml of fluoresceinated SPA (protein concentration 250 μg/ml). The cells were then washed three times with cold PBS pH 7·4 and resuspended with a drop of PBS containing 50% glycerine. Half a drop of the cell suspension was placed on a slide and overlayed with a cover slip.

For the detection of cells able to form rosettes with StaCw, 0·1 ml of medium containing 5×10^7 fluorescent StaCw were mixed with 0·1 ml of medium containing 1×10^6 lymphocytes. The mixture was centrifuged at 200 *g* for 10 min and incubated at 4° for 1 h before mounting on the slide.

Detection of lymphocyte surface markers

E-rosette-forming cells (E-RFC) and Ig-bearing lymphocytes were detected by techniques previously reported (Romagnani, Maggi, Amadori, Giudizi & Ricci, 1977).

For the detection of EA-rosette-forming cells (EA-RFC), an anti-ox erythrocyte rabbit antiserum was used. The antiserum was inactivated at 56° for 30 min and fractionated on a DEAE cellulose column. Washed ox erythrocytes (4×10^8/ml) were incubated with an equal volume of the purified IgG solution at a concentration of 200 μg/ml for 30 min at room temperature. Ox red blood cells (ORBC) were then washed three times with PBS and resuspended at a concentration of 1×10^8/ml. Lymphocytes ($0{\cdot}3 \times 10^6$ in 0·1 ml of medium) were mixed in a plastic tube (60×10 mm) with 0·1 ml of the ORBC suspension. The cells were centrifuged for 5 min at 200 *g*, gently resuspended and counted in a haemocytometer.

Detection of phagocytic cells

Phagocytic cells were detected by neutral red staining, according to the technique described by Arnaiz-Villena, Gyöngyossy & Playfair (1974).

Preparation of tonsil and blood lymphocytes and separation procedures

Tonsil and blood lymphocytes were prepared and separated by E-rosetting essentially as reported

elsewhere (Romagnani *et al.*, 1977). Briefly, mononuclear cell suspensions recovered from the interface of a Ficoll-Hypaque gradient followed by depletion of phagocytes by magnetism, were E-rosetted with neuraminidase-treated sheep red blood cells (SRBC). E-RFC were separated from non E-RFC on a Ficoll-Hypaque density gradient. The E-RFC in the pellet were resuspended and further separated on a second density gradient. This double-step procedure gave suspensions containing 96–98% E-RFC. SRBC were lysed by 0·87% NH_4Cl. Suspensions virtually free from E-RFC were obtained by re-rosetting the cells recovered at the interface of the Ficoll-Hypaque gradient and centrifuging on another density gradient.

EA-rosetting was performed by incubating 1 ml of the lymphocyte suspension (3×10^6/ml) with an equal volume of ORBC (4×10^8/ml) sensitized with IgG anti-ORBC. After centrifugation on a Ficoll-Hypaque density gradient, the interface and pellet cell populations were collected. ORBC were lysed by hypotonic shock with distilled water for half a minute followed by restoring osmolarity with 0·3 M NaCl.

When required, adherent cells were removed according to the method described by Greaves, Roitt, Zamir & Carnaghan (1967). Cell suspensions were incubated on a glass column filled with 50–80 mesh styrene divinylbenzene copolymer beads (Dow Chemical) in medium containing 20% FCS. After 30 min incubation at 37° the cells were eluted with warm medium at a flow rate of 0·5 ml/min.

In vitro cultures

The technique of *in vitro* cultures has been described in detail in previous papers (Romagnani, Amadori, Biti, Bellesi & Ricci, 1976; Romagnani *et al.*, 1977).

Stimulators

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 coupled to Sepharose beads at concentrations ranging between 0·005 and 5 mg/ml. PHA (Difco) was dissolved with 5 ml of medium and used at concentrations ranging between 0·2 and 200 μl/ml. PPD (Weybridge), and Candida (Hollister-Stier), were used at concentrations ranging between 0·1 and 100 μg/ml and 2 and 200 μl/ml, respectively.

RESULTS

Identification of cells marked by fluorescent StaCw

Fluorescent StaCw were found to be able to react with membrane constituents of a small number of peripheral blood mononuclear cells and to form typical rosettes (range 1–9%; mean value: $4{\cdot}1 \pm 0{\cdot}1$). An equivalent number of stained cells (mean value: $3{\cdot}2 \pm 0{\cdot}5$; range 0·3–8·3) was observed when lymphocytes were incubated with fluoresceinated SPA.

By separation procedures it was possible to demonstrate that purified T lymphocytes did not bind fluorescent StaCw, whereas the number of cells forming rosettes with StaCw increased on the average up to 15% in the cell population recovered at the interface after the E-rosetting. When the cell suspension recovered at interface of E-rosetting was further fractionated by filtration on a styrene bead column, it was shown that the great majority of cells capable of forming rosettes with StaCw are adhering cells. After incubation of blood mononuclear cells for 1 h at 4° with aggregated human IgG (Agg–IgG), the number of cells able to form rosettes with StaCw constantly increased up to 10% or more. These values were similar to those which could be shown when the same populations were tested for their capacity to form EA rosettes with ox erythrocytes sensitized with anti-ORBC rabbit IgG. An increase of labelled cells was also seen, even though less constantly, when lymphocytes were preincubated with soluble IgG, human or rabbit serum stored at −20°. On the contrary no increase could be observed when the cells were preincubated with soluble or aggregated human IgM, nor with FCS or foetal calf gammaglobulins.

The inability of calf proteins to react with StaCw constituents was confirmed by further experiments. Tanned SRBC, sensitized with human gammaglobulin, formed rosettes with fluorescent StaCw, whereas SRBC sensitized with foetal calf gammaglobulins did not. Moreover, when soluble SPA was tested in immunodiffusion with myeloma IgG or human serum, a marked precipitin line was observed. On the contrary, no precipitin lines could be detected when soluble SPA was tested with the same concentrations of foetal calf gammaglobulin or foetal calf serum. These observations appeared of interest in view of the cell activation experiments.

In vitro response to StaCw of lymphocytes cultured in AB serum, medium alone or foetal calf serum

Blood or tonsil lymphocytes stimulated with StaCw in the presence of AB human serum showed a scarcely significant incorporation of tritiated thymidine at day 3, even though it was higher at day 6. When the cells were cultured in medium alone, as suggested by Forsgren, Svedjelund & Wigzell (1976), a marked proliferation was shown also at day 3, but the results were frequently affected by high background values, especially if purified populations were assayed in culture.

Thus, we tried to stimulate human lymphocytes in the presence of 10% foetal calf serum and we were able to show a significant and reproducible proliferative response at day 3. In this way we could study the response of purified lymphocyte subpopulations in a more standardized system.

In vitro response of purified cell populations to StaCw

When purified T lymphocytes were isolated from peripheral blood by E-rosetting, they were unable to respond *in vitro* to StaCw, whereas the response of the non-T-cell rich interface population was significantly greater than that of the unfractionated population (Fig. 1).

By rosetting peripheral blood lymphocytes with ORBC coated with anti-ORBC rabbit IgG, EA-RFC could be separated from other lymphocytes. Non rosetting cells responded *in vitro* to StaCw, whereas lymphocytes which formed EA-rosettes did not (Fig. 2).

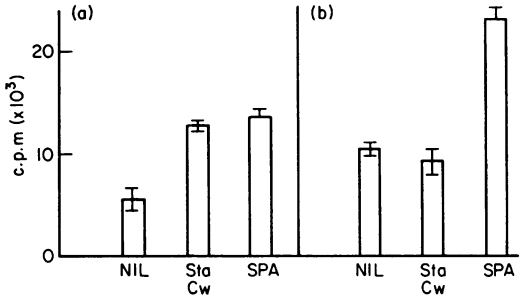

Figure 2. *In vitro* response to StaCw and soluble SPA of peripheral blood EA-RFC and EA-RFC-depleted lymphocytes. (a) Interface. (b) Pellet.

In order to investigate whether or not the response of B-cell enriched populations to StaCw needed the presence of contaminating T cells, lymphocyte suspensions from human tonsils were virtually depleted of E-RFC by re-rosetting the interface cells obtained by a single E-rosetting. Thus, it was possible to show that T-cell depleted populations, in which the number of contaminating T cells was reduced to less than 1%, were still able to respond *in vitro* to StaCw. The addition of increas-

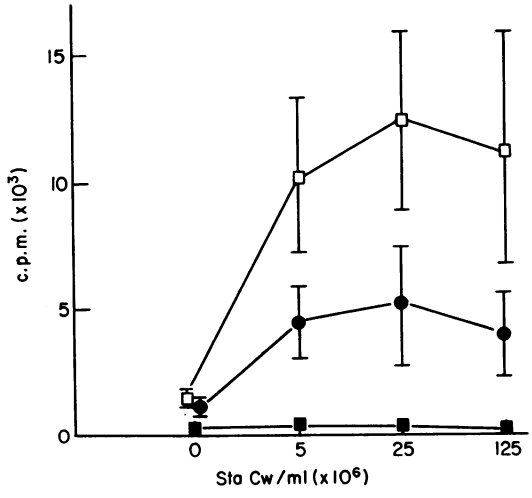

Figure 1. *In vitro* response to StaCw of unseparated (●); B-cell enriched (□); and B-cell depleted (■) peripheral blood lymphocytes.

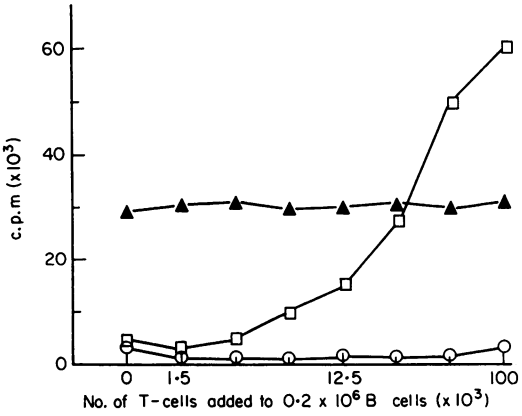

Figure 3. Effect of addition of increasing concentrations of autologous T lymphocytes on the response to medium alone (○), to StaCw (▲) and PHA (□) of cultures containing highly purified B lymphocytes from human tonsils. Highly purified B lymphocytes were obtained by re-rosetting interface cells obtained after a single E-rosetting with neuraminidase-treated SRBC.

ing amounts of T cells to the T-cell depleted suspension did not induce any increase of the thymidine uptake (Fig. 3).

In vitro response of purified cell populations to soluble SPA

In our culture system, soluble SPA was also able to induce a very significant incorporation of tritiated thymidine by human lymphocytes. However, marked differences were noted between the stimulating capacity of StaCw and soluble SPA.

When the lymphocyte response was studied before and after depletion of phagocytic cells by magnetism it was possible to show that monocyte-depleted cell suspensions responded to StaCw better than undepleted suspensions, whereas the response to soluble SPA was depressed by the depletion of phagocytic cells.

Moreover, highly purified T lymphocytes from peripheral blood were still able to show a small but significant proliferative response to soluble SPA, particularly when high concentrations were used. On the other hand, the proliferative response of B-cell enriched populations was less marked than that of unfractionated populations (Table 1).

Either EA-RFC or suspensions depleted in EA-RFC showed a significant response (Fig. 2).

Finally, when B-cell enriched suspensions from tonsils, containing less than 1% E-RFC, were stimulated with soluble SPA, they did not show any significant proliferation (Table 2). However, the addition to B-cell rich suspensions from tonsils of autologous E-RFC was able to restore a marked incorporation of ^{3}H-thymidine. A significant response, even though less marked, could be also demonstrated in these cultures when autologous mitomycin-treated T lymphocytes were added (Fig. 4). Moreover, the response of highly purified E-RFC from human blood could be potentiated by

Table 1. *In vitro* response to soluble SPA of unseparated and B-cell depleted peripheral blood lymphocytes

	Stimulant			
	Soluble SPA (μg/ml)			
Cell population	None	5	25	125
Unseparated	1600 ± 639	24,737 ± 6451	37,413 ± 11,511	31,695 ± 9341
B-cell depleted*	204 ± 63	543 ± 211	1303 ± 418	1785 ± 715

* Highly purified T cells were obtained by re-rosetting the pellet population recovered after a single E-rosetting. Contaminating non-T cells were less than 1%. The values indicate the mean ± S.E.M. of three separate experiments.

Table 2. *In vitro* response to soluble SPA of unseparated and T-cell depleted tonsil lymphocytes

	Stimulant			
	Soluble SPA (μg/ml)			
Cell population	None	5	25	125
Unseparated	4691 ± 1460	51,628 ± 7432	55,624 ± 2836	51,269 ± 1834
T-cell depleted*	914 ± 133	1211 ± 610	1266 ± 556	1426 ± 835

* Highly purified B cells were prepared by re-rosetting the interface population obtained after a single E-rosetting. Contaminating E-RFC were less than 1%. The values indicate the mean ± S.E.M. of three separate experiments.

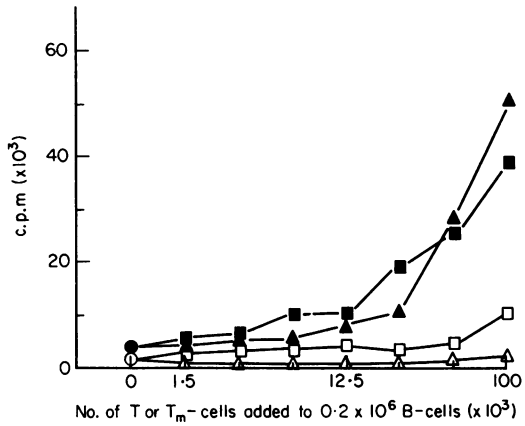

Figure 4. Effect of addition of increasing concentrations of untreated (T) and mitomycin-treated (T_m) autologous T lymphocytes on the response to soluble SPA of cultures containing highly purified B lymphocytes from human tonsils. The cells were incubated with medium alone (open symbols) or soluble SPA, 25 μg/ml (closed symbols).

the addition of either untreated or mitomycin-treated autologous B-cell rich suspensions.

Comparison between the mitogenic activity of soluble and insoluble SPA

In order to determine whether the different mitogenic activity of StaCw and soluble SPA was due to the presence on StaCw of a component other than SPA or to a particular presentation of SPA to the lymphocytes when attached to an insoluble matrix, highly purified T and B lymphocytes from human tonsils were stimulated with SPA covalently linked to Sepharose beads. Covalently linked SPA, like StaCw, was unable to stimulate highly purified E-RFC, but it activated very well the B-cell enriched suspensions in which the number of contaminating E-RFC was reduced to less than 1% (Table 3).

In vitro response to soluble SPA and StaCw of umbilical cord blood lymphocytes

Umbilical cord lymphocytes stimulated with soluble SPA or StaCw showed a strong proliferative response at day 3. In contrast some antigens, such as PPD or Candida, produced no response at day 3 and induced no significant thymidine incorporation even at day 6.

DISCUSSION

Until now little evidence has been presented on the reliability of B-cell mitogens in man. It is well known that T-cell mitogens, such as PHA and PWM, can also activate B cells, but this activation is always a T-cell mediated phenomenon. As few as 1–5% contaminating T lymphocytes are able to restore a significant thymidine incorporation by B-cell rich population stimulated with PHA (Janossy and Greaves, 1975; Weksler & Kunz, 1976; Romagnani *et al.*, 1977). LPS, which is a B-cell mitogen in rodents, does not work well in man. Anti-Ig antibodies are unable to stimulate suspensions of B lymphocytes when they are completely depleted of T cells (Gausset, Delespesse, Hubert, Kennes & Govaerts, 1976). It has been reported that anti-human β_2 microglobulin is mitogenic for B lymphocytes in man (Ringden & Möller, 1975), but it was not ascertained whether it was also able to activate B-cell suspensions totally depleted of T cells. Recently, a T-cell independent B-cell mitogen has been extracted from Nocardia (Brochier, Bona, Ciobaru, Revillard & Chedid, 1976).

Table 3. The mitogenic activity of StaCw, soluble and insoluble SPA on B cell-enriched and B cell-depleted populations from human tonsils

Cell population	Stimulant				
	None	StaCw (25×10^6/ml)	SPA-Seph. (0·5 mg/ml)	SPA (5 μg/ml)	PHA (10 μl/ml)
Unseparated	4243 ± 1214	21,860 ± 4215	27,948 ± 5018	50,563 ± 9318	90,577 ± 13,210
B cell-enriched	1799 ± 1343	25,871 ± 4738	23,060 ± 4824	3079 ± 712	3809 ± 649
B cell-depleted	2826 ± 948	3566 ± 946	3769 ± 1011	15,192 ± 2413	81,098 ± 12,470

The values indicate the mean ± S.E.M. of three separate experiments.

The results of this paper confirm the data previously reported by Forsgren *et al.* (1976), who showed that human peripheral blood non-T cells are activated by StaCw, whereas purified T cells are not. They also demonstrate that the activation of non-T cells does not need the presence of contaminating T cells. In addition, they strongly suggest that the non-T cells which proliferate in the presence of StaCw are B lymphocytes. One could thus conclude that StaCw are a selective T-cell independent B-cell mitogen in man.

The mitogenic activity of soluble SPA was found to be different from that of StaCw. This difference could be explained by suggesting that the activation of B cells by StaCw is due to a bacterial constituent different from SPA. Kreger, Cuppari & Taranta (1972) demonstrated that two extracellular components isolated free of detectable amounts of SPA from staphylococcal culture filtrates were mitogenic for human lymphocytes. On the other hand, Forsgren *et al.* (1976) showed that cell wall constituents other than SPA, like mucopeptide or teichoic acid, are unable to activate human lymphocytes. When we tried to stimulate human lymphocytes with SPA linked to Sepharose beads, we found that insoluble SPA, like StaCw, is able to activate highly purified B lymphocytes from tonsils and induces little or no mitogenic activity in purified T lymphocytes. Thus, one can speculate that soluble SPA acts as an antigen on purified T lymphocytes and can stimulate B cells only in the presence of T cells. When attached to an insoluble matrix, it does not activate T cells, but is able to interact directly with B cells.

The exact nature of B-cell subpopulation capable of responding to StaCw and insoluble SPA, and the mechanism by which these stimulants exert their mitogenic activity are unknown. Forsgren *et al.* (1976) suggested that the mitogenicity of SPA could be due to a selective binding to the surface IgG molecules of B cells. Our results seem to suggest that the cells which passively absorb IgG by their receptor for the Fc fragment are not responsible for the *in vitro* response to StaCw. In addition, it has been recently shown that peripheral blood B lymphocytes with intrinsic membrane IgG are less than 1% of the population (Winchester *et al.*, 1975). It is difficult to think that so few cells are responsible for the PHA-like response induced by SPA at day 3. Unfortunately, a definite conclusion concerning the identity of cells reacting with fluorescent StaCw could not be drawn from the results of experiments described here. In fact, the number of StaCw-RFC-was usually higher than 1% of the total population. Thus, it may be suggested that the cells which are able to form StaCw rosettes are not exclusively B lymphocytes with intrinsic IgG or, alternatively, that a mechanism other than the binding of StaCw to the surface IgG molecules of B cells is also responsible for the *in vitro* response.

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