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T-cell independence of immunoglobulin synthesis by human peripheral blood lymphocytes stimulated with SpA-containing staphylococci

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Summary. Unfractionated and T-cell depleted human peripheral blood lymphocytes (PBL) were cultured *in vitro* in the presence of pokeweed mitogen (PWM) and *Staphylococcus aureus* strain Cowan I (StaCw). After 7 days of culture, the cells were assayed for cytoplasmic immunoglobulins (Cyto-Ig) by direct staining using fluorescein-labelled $F(ab')_2$ fragments prepared from specific antisera against human IgG $F(ab')_2$. The amount of immunoglobulin of the IgM and IgG class released into the cell-free supernatants was also measured by radioimmunoassay. In unfractionated PBL StaCw, like PWM, was able to induce a significant increase of either the number of Cyto-Ig containing cells or the amount of IgM and IgG secreted into the supernatant. In contrast, the amount of IgM and IgG immunoglobulin released into the supernatant of T-cell depleted suspensions stimulated with PWM was significantly reduced in comparison with that of unfractionated populations, whereas it was unchanged in T-cell depleted *vs* unfractionated suspensions stimulated with StaCw. The addition of a few T lymphocytes restored the ability of T-cell depleted suspensions to produce Ig in the presence of PWM, whereas despite addition of high numbers of T cells no further augmentation of the Ig production induced by StaCw on T-cell depleted suspensions was observed. Cultures of umbilical cord blood lymphocytes (UCBL) stimulated with PWM did not generate Ig-producing cells, whereas UCBL stimulated with StaCw showed significant production of Ig of both IgM and IgG classes. The results indicate that T lymphocytes are probably not involved either with stimulation or with the suppression of Ig production induced by StaCw.

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INTRODUCTION

Most substances, well characterized as polyclonal B-cell activators (PBA) for murine spleen cells (Coutinho & Möller, 1975), are inactive as such, when tested on human lymphocytes. Recently, however, anti β_2-microglobulin, LPS, a *Nocardia opaca* extract and some bacteria or viruses were demonstrated to induce antibody production in human spleen and/or peripheral blood cells (Ringdén & Möller, 1975; Ringdén, Rynnel-Dagöö, Waterfield, Möller & Möller, 1977; Luzzatti, Hengartner & Schreier, 1977; Ringdén & Rynnel-Dagöö, 1978; Lethibichtuy, Ciorbaru & Brochier, 1978; Banck & Forsgren, 1978).

In a previous paper, we showed that *Staphylococcus aureus* strain Cowan I (StaCw) is a human T-cell independent B-cell mitogen (Romagnani, Amadori, Giudizi, Biagiotti, Maggi & Ricci, 1978). In the present study, the synthesis and secretion of immunoglobulins (Ig) by unfractionated and T-cell depleted human peripheral blood lymphocytes (PBL) cultured *in vitro* in the presence of pokeweed mitogen (PWM)

and StaCw was evaluated. The effect of these mitogens on Ig synthesis by umbilical cord blood lymphocytes (UCBL) was also investigated.

MATERIALS AND METHODS

Preparation of cells

Lymphocytes were obtained from the peripheral blood of adult subjects or from umbilical cord blood and purified as reported elsewhere (Romagnani *et al.*, 1978). Briefly, mononuclear cell suspensions recovered at the interface of the Ficoll–Hypaque density gradient were depleted of phagocytes by incubation with carbonyl iron followed by removal of cells which had ingested the metal with a magnet. The phagocyte-depleted cells were washed and E-rosetted with neuraminidase-treated sheep red blood cells. E-rosette forming cells (E-RFC) were separated from non E-RFC by centrifugation on a density gradient. Suspensions virtually free from T lymphocytes were obtained by multiple sequential E-RFC depletions. After three cycles of E-RFC depletion almost all of the T cells had been eliminated from the cell preparation ($< 1\%$ E-RFC). Contaminating monocytes were identified by non-specific esterase stain (Koski, Poplak & Blaese, 1975). The number of esterase-staining cells found in non-T-cell populations initially depleted of phagocytic cells with carbonyl iron ranged from 2 to 10%. These non-T fractions from peripheral blood contained primarily Ig^+ cells and will subsequently be referred to as B-cell fractions.

Culture conditions

Unfractionated lymphocytes or B-cell fractions were suspended in RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated foetal calf serum (FCS; Gibco, Grand Island). Each culture consisted of 1 ml of medium containing 2×10^6 lymphocytes in 17×100 mm tissue culture tubes (Falcon Plastic, Oxnard). Different concentrations of PWM (GIBCO) or StaCw were added to cultures. Cultures were then incubated at 37° in a 95% air–5% CO_2 humidified atmosphere for 7 days.

Stimulators

PWM was used at the concentration of 5 μl/ml. StaCw strains were originally obtained from the National Collection of Type Cultures (London). The bacteria were killed by incubation in 0·5% formaldehyde for 3 h at room temperature and heat-treated at 80° for 3 min, washed and finally stored at −80°. They were used at concentrations ranging between 1 and 25×10^6 bacteria/ml diluted in 10% FCS-supplemented medium in round-bottommed tubes.

Antisera

Antisera against human IgG $F(ab')_2$ and μ chain were prepared in rabbits and purified as described previously (Romagnani, Almerigogna, Giudizi & Ricci, 1980a). Briefly, anti-$F(ab')_2$ antisera were obtained by injecting rabbits with $F(ab')_2$ fragments prepared from human IgG by treatment with pepsin (2%) in acetate buffer pH 4·0 for 18 h at 37°. The digested material was filtered on Sephadex G-150 and SpA-Sepharose CL-4B to remove undigested IgG. Anti-$F(ab')_2$ antibodies were purified by adsorption and elution from a Sepharose–4B-$F(ab')_2$ column with glycine–HCl buffer, pH 2·5. $F(ab')_2$ fragments were prepared from anti-$F(ab')_2$ immunosorbent-purified rabbit antibodies by treatment with pepsin, as described above.

For anti-μ antibodies, rabbits were injected with IgM purified by repeated precipitation with distilled water and Sephadex G-200 gel filtration from pooled sera of macroglobulinaemia patients. The antiserum was absorbed with Sepharose-4B beads covalently linked with cord blood gammaglobulins, normal serum IgG, $F(ab')_2$ fragments, an IgA and an IgD myeloma protein. After these absorptions purified anti-μ antibodies were absorbed and eluted from a Sepharose-4B-IgM column with glycine–HCl buffer, pH 2·5.

Specificity of the purified antibodies was established by immunoprecipitation in agarose gels. Conjugation of FITC (Sigma) to the reagents was performed as previously reported (Romagnani *et al.*, 1978). In the case of the anti-μ antiserum the specificity was further checked by testing with a panel of monoclonal marrow plasma cells.

Assay for detection of cytoplasmic Ig (Cyto-Ig)

After 7 days of incubation at 37°, unstimulated and stimulated cultures were harvested by centrifuging the cells for 15 min at 400 ***g*** and the cell pellets were washed three times in cold RPMI medium. To remove bacteria, the cell pellets from cultures stimulated with StaCw were layered over 2 ml of undiluted FCS in 12×75 mm plastic tubes and spun at 200 ***g*** for 10 min at 4°. Washed lymphocytes (2×10^5 cells in 0·1 ml) were layered over a microscope slide in a cytocentrifuge. Slides were air-dried, fixed in ethanol (95%)—acetic acid (5%) at −20° for 15 min and

washed three times in phosphate-buffered saline, pH 7·4 (PBS). Fixed cells were incubated with fluorescein-labelled $F(ab')_2$ fragments for 30 min at room temperature. Slides were washed three times in PBS, mounted with glycerol–PBS, pH 7·8 and examined using a Leitz microscope. The relative number of Cyto-Ig containing cells was evaluated by counting 1000 cells.

Assays for detection of IgM and IgG in the culture supernatants

IgM secreted into the culture medium of day 7 lymphocyte cultures was detected by a paper disc radioimmunoassay using solid phase-coupled anti-human μ antibodies and immunosorbent-purified ^{125}I-labelled anti-μ antibodies. Paper discs (diameter 5·5 mm; weight 2·3 mg) were prepared from ashless paper and activated with CNBr according to the method of Ceska & Lundkvist (1972). The IgG fraction obtained by DEAE chromatography from rabbit antiserum against human μ chain was coupled to the discs according to the method reported by Ceska & Lundkvist (1972). Immunosorbent-purified anti-μ antibodies were labelled with ^{125}I by the chloramine-T method (Hunter & Greenwood, 1962). The assay was performed in two steps. During the first incubation period (at least 12 h), the IgM present in the test sample or in the standard was allowed to bind to the antibody-coated disc. During the second incubation period (at least 12 h), radioactive antibodies were allowed to react with the bound IgM. After three washings, the radioactivity on the disc was counted in a gamma-counter. A standard curve was obtained using human IgM purified as described above. Either standard IgM or test samples were diluted in PBS containing 2% FCS.

IgG was detected in the supernatants using a radioimmunoassay system based on the ability of IgG to inhibit the binding of radio-iodinated Staphylococcal protein A (SpA) to IgG linked to a solid phase (Romagnani, Del Prete, Giudizi, Almerigogna & Ricci, 1979). Briefly, 0·1 ml of test sample was incubated with 0·1 ml of ^{125}I-SpA at 30° for 1 h in disposable plastic tubes. 0·1 ml of a suspension of ox red blood cells coated with an anti-ox red blood cells rabbit IgG ($E_{ox}A_G$) was then added and the mixture further incubated at 30° for 1 h. The tubes were washed three times with PBS and the radioactivity incorporated in the pellet was determined by counting in a gamma-counter. A standard inhibition curve was obtained by determining the c.p.m. displaced from the ^{125}I-SpA-$E_{ox}A_G$ complex by different concentrations of human purified IgG. The amount of IgG secreted by the cells into the culture supernatants was measured by comparing the percentage inhibition with the standard inhibition curve. Samples for both IgM and IgG assays were prepared by again centrifuging cell-free supernatants at 2000 *g* for 30 min at 4°.

RESULTS

Ig production by unfractionated PBL stimulated with StaCw

Augmentation of either the number of Cyto-Ig containing cells or the amount of IgM and IgG released into the supernatants was observed in day 7 cultures of PBL from twenty-eight adult healthy individuals both in the presence of PWM and StaCw (Table 1). The number of Cyto-Ig containing cells was the same or slightly lower in cultures stimulated with PWM than in those stimulated with StaCw. However, the intensity of staining was usually greater in PWM-stimulated cultures. Peak responses were usually obtained with bacterial concentrations ranging from 1 and 25×10^6/ml.

Effect of T-cell depletion on the Ig production induced by StaCw

The necessity for T-cell help in the Ig production induced by StaCw was investigated. This was done by attempting to remove as many T cells as possible from PBL by the E-rosetting technique. To ensure the com-

Table 1. Comparison of Ig production in cultures of PBL stimulated with PWM and StaCw

Stimulant	Cyto-Ig containing cells (%)	Supernatant Ig† (ng/2 × 10^6 cells) IgM	IgG
None	1·5±0·2	572±116	805± 94
PWM	8·1±0·8	1755±262	2613±237
StaCw	10·0±0·8	1844±317	3782±582

*Percentage of cells positive for cytoplasmic Ig measured by direct immunofluorescence with $F(ab')_2$ fragments against human $F(ab')_2$ on day 7 of culture.

†Amount of Ig detected by radioimmunoassays in day 7 culture supernatants.

The results represent the mean value (±SE) of twenty-eight separate experiments.

Table 2. Stimulation of IgM and IgG synthesis by PWM and StaCw in 7 day cultures of unseparated and T-cell depleted PBL

Cell population	Stimulant	Supernatant Ig (ng/2 × 10^6 cells) IgM	IgG
Unfractionated	None	572 ± 116	805 ± 94
	PWM	1755 ± 262	2613 ± 237
	StaCw	1844 ± 317	3782 ± 582
T-cell depleted*	None	422 ± 97	1702 ± 340
	PWM	662 ± 260	1326 ± 147
	StaCw	1608 ± 291	3075 ± 506

*T lymphocytes were removed by three cycles of separate incubations with neuraminidase-treated sheep red blood cells and centrifugation through a density gradient. In all the experiments T-cell depleted populations comprised of < 1% E-RFC.

The results represent the mean value ± SE of thirteen separate experiments.

pleteness of this removal, multiple sequential E-RFC depletions were carried out. After three cycles of E-RFC depletion almost all of the T-cells had been eliminated from the cell preparation (< 1% E-RFC) in thirteen experiments. As shown in Table 2 the amount of IgM and IgG released into the supernatants of PWM-stimulated cultures, as detected by radioimmunoassays, was dramatically reduced by T-cell depletion, whereas no evidence of reduction was found in StaCw-stimulated B-cell cultures. On the other hand, the addition of 2 × 10^6 B cells of as few E-RFC as 10 × 10^4 was sufficient to restore the Ig production induced by PWM. In contrast, the addition of up to 2 × 10^6 E-RFC to the B-cell fractions did not induce any significant increase of the amount of IgM and IgG released into the supernatant of StaCw-stimulated cultures (Fig. 1).

Ig production by UCBL stimulated with StaCw

Ig production induced by PWM in cultures of UCBL from four newborns was virtually absent, as detected by evaluating the number of Cyto-Ig containing cells (1·5 ± 0·3% *vs* 2·1 ± 0·4%). In contrast, in the same cases StaCw-stimulated cultures showed a significant increase in the number of Cyto-Ig containing cells (13·5 ± 3·0% *vs* 2·1 ± 0·4%). The amount of IgM and IgG released into the supernatants was also evaluated in cultures of UCBL from these, as well as five other newborns. A significant amount of IgM was found in the supernatants of StaCw-stimulated UCBL cultures from 6 out of 9 newborns, even though at lower levels than those found in StaCw-stimulated PBL cultures from adult individuals. In addition, a significant amount of IgG was also detected in the supernatants of StaCw-stimulated UCBL cultures from 4 out of 9 newborns (Fig. 2).

DISCUSSION

It has been shown that some strains of *Staphylococcus aureus* are capable of inducing DNA synthesis in human lymphocytes (Forsgren, Svedjelund & Wigzell,

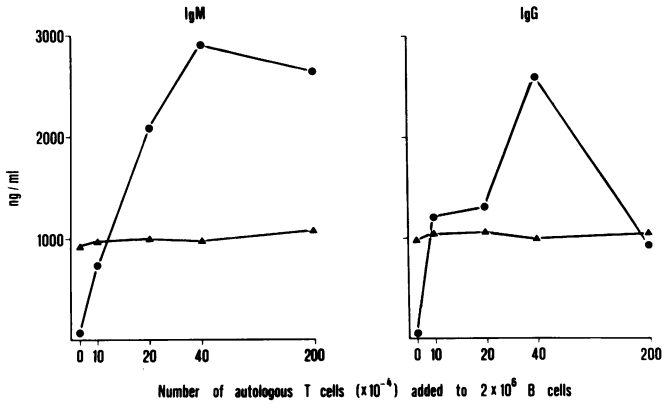

Figure 1. Effect of addition of different concentrations of autologous T cells on the IgM and IgG synthesis by B cells stimulated with PWM (circles) and StaCw (triangles). The results are expressed in ng/ml in stimulated cultures minus ng/ml in unstimulated cultures and represent the mean value of two separate experiments.

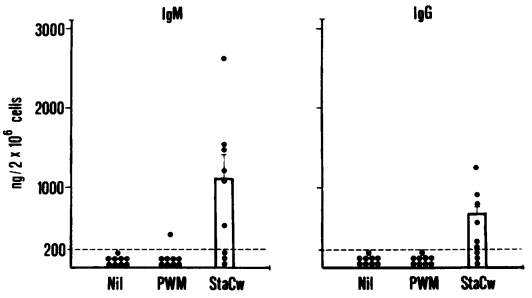

Figure 2. Detection of IgM and IgD in 7 day supernatants of UCBL cultures stimulated with PWM and StaCw.

1976; Ringdén *et al.*, 1977; Romagnani *et al.*, 1978; Banck & Forsgren, 1978). The ability of *S. aureus* strains to stimulate antibody production *in vitro* has also been investigated in both humans and mice. StaCw was found to be capable of inducing intracellular Ig synthesis in human splenic but not peripheral blood lymphocytes (Möller & Landwall, 1977). However, in further experiments, these, as well as other, bacteria were shown to stimulate polyclonal antibody secretion in human blood lymphocytes (Ringdén *et al.*, 1977; Rynnel-Dagöö *et al.*, 1978; Sjöberg & Kurnick, 1980; Pryjma, Munoz, Galbraith, Fudenberg & Virella, 1980a; Pryjma, Munoz, Virella & Fudenberg, 1980b).

The results in this paper confirm that human PBL can be triggered to differentiate into either IgM or IgG antibody-producing cells by appropriate concentrations of StaCw. In addition, a significant difference between the activating capacity of PWM and StaCw was observed. At the concentrations used in this study, StaCw, unlike PWM, were found to induce antibody synthesis and secretion in T-cell depleted lymphocyte cultures. The finding that PWM-induced antibody production by human B cells requires T-cell help is in agreement with a number of previous observations (Keightley, Cooper & Lawton, 1976; Fauci, Pratt & Whalen, 1976; Hirano, Kuritani, Kishimoto & Yamamura, 1977; Saxon, Stevens & Ashman, 1977). In contrast, the role of T cells in the antibody production induced by StaCw is more controversial. Ringdén *et al.* (1977) found a significantly lower number of plaque-forming cells in purified B-cell suspensions than in unfractionated lymphocytes or mixtures of purified B and T cells. However, using the plaque-forming cell response against sheep red blood cells, it is difficult to demonstrate the degree of dependence on T cells, since the removal of T cells by the E-rosetting procedure may also remove B lymphocytes equipped with Ig receptors for sheep red blood cells (Sjöberg & Kurnick, 1980). More recently, by enumeration of cells containing Cyto-Ig and by quantification of Ig released into culture supernatants by laser nephelometry, Pryjma *et al.* (1980a, b) showed that StaCw were also active in cultures depleted of T cells.

Our results are in agreement with those reported by Pryjma *et al.* (1980a, b). In fact, after exhaustive depletion of T cells by multiple sequential E-rosetting steps, StaCw-stimulated, unlike PWM-stimulated, peripheral blood B cells still produced significant amounts of Ig of the IgM and IgG classes. In addition, the capacity of such rigorously T-cell depleted B-cell populations to produce Ig in the presence of StaCw was not augmented when different concentrations of T lymphocytes were added back to the cultures. In contrast, the addition of a few E-RFC was able to restore the Ig production by T-cell depleted B-cell suspensions stimulated with PWM.

Further differences between PWM and StaCw were demonstrated in experiments in which the ability of UCBL to produce Ig was evaluated. In agreement with results previously reported by other workers (Hayward & Lawton, 1977; Durandy, Fischer & Criscelli, 1979; Miyawaki, Seki, Kubo & Taniguchi, 1979; Morito, Bankhurst & Williams, 1979), we found that cultures from newborn lymphocytes stimulated with PWM, did not generate Ig-producing cells. This defective B-cell maturation has been attributed to an active suppression mediated by T lymphocytes (Durandy *et al.*, 1979; Miyawaki *et al.*, 1979; Morito *et al.*, 1979). The results reported in this paper show that, unlike PWM, StaCw are able to generate Ig-producing cells in UCBL cultures and to induce these cells to secrete Ig not only of the IgM class but also of the IgG class. These findings indicate that the stimulatory effect of StaCw is resistant to the influence of the newborn T-cell suppressor activity and, therefore, they may be consistent with the results of Pryjma *et al.* (1980a, b) and of Kurnick, Pandolfi & Wigzell (1980), showing that the stimulatory effect of StaCw was resistant to the influence of suppressor T cells generated by co-stimulation with concanavalin A or by pre-incubation without mitogenic stimulus. Thus, some data suggest that T lymphocytes are probably not involved either with the stimulation or with the suppression of Ig production induced by StaCw.

The possible explanations for the observed differences in Ig production between cultures stimulated with PWM and with StaCw remain speculative

because the mechanism by which StaCw exerts its activity on B cells is still unknown. It has been shown that SpA in soluble form or coupled to Sepharose also acts as PBA for human splenic and peripheral blood lymphocytes (Ringdén & Rynnel-Dagöö, 1978). In addition, in previous experiments we demonstrated that the proliferative response of tonsil B cells to StaCw is mainly due to a subset of IgM- and/or IgD-bearing lymphocytes capable of forming rosettes with SpA-coated erythrocytes (Romagnani, Giudizi, Almerigogna & Ricci, 1980c; Romagnani, Giudizi, Almerigogna, Nicoletti & Ricci, 1980b). More recently, we were also able to show that unfractionated, as well as T-cell depleted peripheral blood lymphocytes from some patients with chronic lymphocytic leukemia can incorporate significant amounts of [^{3}H]-thymidine after stimulation with SpA-containing Staphylococci. This proliferative response was peculiar to patients whose lymphocytes were able to form rosettes with SpA-coated erythrocytes (Romagnani, Giudizi, Almerigogna, Biagiotti, Bellesi, Bernardi & Ricci, 1980d). It cannot obviously, be excluded that there are other substances than SpA on Staphylococcal cells that can activate human B cells, since Staphylococci without SpA also behave as PBA for human B cells (Ringdén *et al.*, 1977). However, there is increasing evidence that StaCw stimulates lymphocytes to proliferate by a signal deriving from interaction of SpA present on the bacterial surface with a membrane component of a subset of IgM- and/or IgD-bearing lymphocytes.

The nature of the membrane component of IgM- and/or IgD-bearing B cells reacting with SpA has not yet been clarified. It could be suggested that SpA binds to IgG which coexist with IgM and presumably with IgD on the surface of a surprising proportion of B lymphocytes (Chiorazzi, Fu & Kunkel, 1980). Another possibility is that the SpA-binding to B cells is due to an interaction between SpA and surface Ig other than IgG, like IgM and/or IgD. Alternatively, it may also be suggested that an interaction with membrane components other than surface Ig is responsible for the binding of SpA to a subset of human B lymphocytes. This possibility is consistent with recent data showing that the PBA property of SpA is not due to its ability to bind to or to cross-link the Fc parts of Ig molecules (Sjödahl & Möller, 1979). However, further work is still necessary in order to elucidate this point, which may contribute to a better understanding of the mechanisms of B-cell activation. Furthermore, the results of the present paper suggest that the simultaneous evaluation of antibody synthesis and secretion induced *in vitro* by PWM and StaCw, T-cell dependent and T-cell independent B-cell activators respectively, may serve as a simple tool in discriminating the deficiencies of antibody production due to B-cell defects from deficiencies in which an alteration of T-cell control is involved.

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