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Mitogenic activity of extracellular cationic products produced by group A streptococci; analysis of the lymphocyte response

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SUMMARY

The cationic fraction (isoelectric point > 8·5) of supernatant products of group A streptococcal cultures exerted a strong mitogenic effect on human peripheral lymphocytes at concentrations as low as 1 ng/well. Incorporation rates were highest at concentrations of 1–10 μg; rabbit peripheral lymphocytes also responded strongly, only a weak response was seen with mouse peripheral lymphocytes and rabbit thymocytes. Purified OKT4 positive (T helper) and OKT8 positive (T suppressor) lymphocyte subpopulations both responded, the former more strongly. Although accessory cells (monocytes) were not absolutely necessary, in their presence higher incorporation of ^{3}H-thymidine was observed. Isolated B cells did not respond.

Keywords mitogenic activity extracellular cationic streptococcal products lymphocyte subpopulations

INTRODUCTION

Mitogenic activity has been reported for various preparations of group A streptococci, including fractions with cationic pI (Cavaillon, Geoffroy & Alouf, 1979; Gray, 1979; Knöll *et al.*, 1983; Seravalli & Taranta, 1974). We have been able to show that cationic extracellular streptococcal antigen (CESA) possesses affinity for the GBM in the rat (Vogt *et al.*, 1985) and that these antigens can be found in glomerular immune deposits in acute post-streptococcal glomerulonephritis (APSGN) in man (Vogt *et al.*, 1983). In this connection it may be relevant that it has been suggested that cellular immune sensitivity to streptococcal antigens is involved in the progression of nephritis (Zabriskie *et al.*, 1970).

The purpose of the present study was to analyse the in-vitro response of peripheral lymphocytes to CESA, to identify the responding lymphocyte subpopulation, and to clarify if help by accessory cells is necessary.

MATERIALS AND METHODS

A group A streptococcal strain (Mar1), isolated from the throat of a patient with APSGN, was cultured in a chemical defined medium (Van de Rijn & Kessler, 1980) for 4 days. The pH of the medium was adjusted to pH 7·0 with 2 M NaOH each day and glucose was added when the culture

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medium was exhausted. The bacteria were spun down and the supernatant fraction sterile-filtered and concentrated in an Amicon cell using a YM10 filter. The concentrated cationic fraction (pI > 8·5) of the preparation was separated by chromatofocussing (cut off technique) on a PBE column (Pharmacia) using an ethanolamine-HCl buffer (pH 8·5) for elution. The first peak was collected and concentrated as mentioned above and after exhaustive dialysis against PBS the antigen was sterile-filtered and stored at 4°C. The protein concentration was determined by the biuret method, using human serum albumin as standard. The molecular weight of this fraction, determined on SDS PAGE electrophoresis and by Sephadex G75 chromatography was in the range of 30,000 daltons.

Experimental methods

Isolation of T-cell subpopulations. The isolation of the mononuclear cell layer was performed using the standard Ficoll-Paque method. The starting volume of human peripheral blood was usually 50 ml.

Nylon-Wool Columns were prepared in a 5 ml syringe with 1·0 g Nylon-Wool (Leuko-pak, Fenwal Laboratories, Illinois 60015, USA), then washed with 80 ml PBS and the same volume of 5% FCS-PBS, both prewarmed at 37°C. The column was pre-incubated for 1 h at 37°C. The mononuclear cells, suspended in 1·0 ml culture medium (10% FCS-RPMI), were then loaded onto the Nylon-Wool column. Immediately afterwards, 0·4 ml cell culture incubated medium was added. The cells were then incubated at 37°C, for 1 h and finally eluted with 15 ml of culture medium (37°C preheated). This was called the T cell-rich fraction; it contained less than 2% monocyte contamination.

Subsequently staphylococcal protein A (SPA) rosetting was performed as previously described (Gronowicz, Coutinho & Melchers, 1976; Uchiyama *et al.*, 1981), with slight modifications. One volume of protein A (0·5 mg/ml), 10 volumes of $CrCl_3$ ($2{\cdot}5 \times 10^{-4}$ M) and 1 volume of packed SRBC (sheep red blood cell), all dissolved in 0·15 M NaCl, were incubated at 30°C for 1 h, washed once with 0·15 M NaCl, and twice with PBS, and then resuspended to 1% SRBC (called SRBC-SPA).

One volume of T cell suspension to be tested (1×10^7/ml) and 1 volume of monoclonal antibody solution (OKT4; 1:50 or OKT8; 1:100) were incubated at 20°C for 30 min, washed three times and suspended in the starting volume of culture medium. Following this step, the cell suspension was mixed with the same amount of 1% SRBC-SPA, centrifuged at 120 ***g*** for 5 min and incubated for 30 min at room temperature.

After gentle resuspension, a Ficoll-Paque gradient separation was performed twice in succession to remove rosetted cells; cells not rosetted (negative selection) were used for stimulation experiments.

Isolation of B cells. To remove the monocytes, KAC-2 (1% 1 μm Silicia gel particles, suspended in PBS, JIMRO, Japan) treatment was performed before the Ficoll gradient-step. Whole blood was supplemented with 10% KAC-2, incubated in a 5% CO_2 atmosphere for 1 h at 37°C, and shaken every 15 min. The Ficoll gradient was performed without dilution. Following T-cell elution from Nylon wool columns as above, the 'B cell-rich fraction' was obtained by eluting with cold PBS and additionally with cold PBS while pressing the Nylon-Wool column with the syringe plunger. To eliminate the T-cell contamination from this cell suspension, SPA rosetting with OKT3 antibody was performed.

S-(2-Aminoethyl-isothiouronium-bromide) (AET) rosetting was also carried out to obtain the B cells. AET (Serva, Feinbiochemica, Heidelberg) was prepared as a 4% aqueous solution and adjusted to a pH of 9·0 with 4 M NaOH. One volume of SRBC was incubated with 100 volumes of AET solution for 15 min at 37°C, and suspended at a concentration of 1% in PBS. After washing three times with 5% BSA-PBS (centrifugation at 400 ***g*** for 10 min), two volumes of the cell suspension to be tested ($2{\cdot}5 \times 10^6$/ml), two volumes of 1% SRBC-AET solution and one volume of FCS (previously absorbed with SRBC) were mixed and incubated for 12 min at 37°C. This mixture was centrifuged at 120 ***g*** for 5 min and then incubated for 15 min at 4°C. A Ficoll-Paque gradient was performed, the white ring was harvested, washed twice and then the cells were counted in a Neubauer chamber.

Isolation of monocytes and panning method. The mononuclear cell fraction from 30 ml of

peripheral blood was separated on a Ficoll-Paque gradient. The cell suspension was incubated on MSP plates to which macrophages bound (dishes were 60 mm diameter, 15 mm deep, coated with inactivated FCS, 2–3 × 10^6 cells per plate). The non-adherent cells were then run through nylon wool columns to separate the B cell-rich and T cell-rich fractions. T cell-rich fractions were then panned on plastic dishes (as above) coated with either OKT4 or OKT8 antibody (diluted 1:100) in a negative selection technique. Monocytes were recovered by addition of 0·2% EDTA in 5% FCS in PBS.

Mitogens and incorporation of ^{3}H-thymidine. The cationic A-streptococcal preparation (CESA) was prepared as described above. A total of 10^5 cells were placed in each well of a microtitre plate, the total volume of culture medium (including test substance) was 200 μl. The mitogens Con A and PWM at concentrations of 2·0 and 0·4 μg per well, respectively, were used as references.

After 72–75 h of incubation in a 5% CO_2 saturated atmosphere ^{3}H-thymidine, 1 μCi/20 μl (specific activity 25 Ci/mM) was added into microwell cultures in triplicate and, 12 h later, the cells were harvested with a Titertec cell harvester.

The incorporation of ^{3}H-thymidine was measured in a liquid scintillation counter.

Effect of antibody on mitogenic stimulation. Cultures were set up as above using 1 μg of CESA per well. One to 20 μl of antiserum was added; two different rabbit antisera raised to the unfractionated cationic fraction (CESA) as well as antiserum to erythrogenic toxin (Statens Serum Institute, Copenhagen) were tested.

Peroxidase-anti-peroxidase method (PAP). The PAP method (Bross *et al.*, 1978) was adopted, in order to check the purity of each cell fraction, using the monoclonal antibodies OKT-3, OKT-4, OKT8, OKT9, OKM1 and Leu10 (Ortho Diagnostics (OKT) and Becton Dickinson (Leu). Three hundred cells were counted per fraction tested.

RESULTS

Mitogenic activity, dose dependency. The CESA exhibited strong mitogenic activity on human peripheral mononuclear cells. Maximal incorporation rates were obtained at concentrations of 1 to 10 μg CESA per well. Significant stimulation (SI ≧ 3·0) was seen with doses as small as 0·1 ng of CESA (Fig. 1).

A comparable response was also observed with rabbit peripheral mononuclear cells when 1 to 100 μg CESA was used, whereas peripheral mononuclear cells from C^{3}H mice responded only very

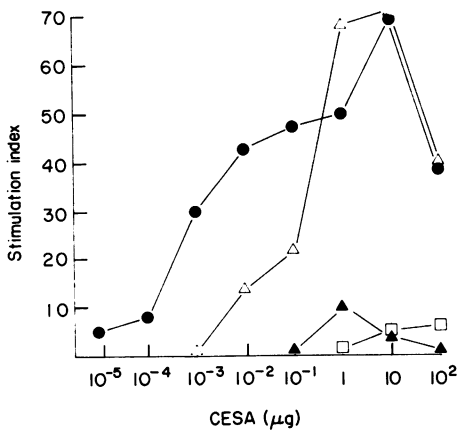

Fig. 1. Stimulatory effect of CESA on lymphocytes of different species. Doses shown are per well (volume 200 μl). Human peripheral mononuclear cells (●——●) were most sensitive to CESA. Rabbit peripheral mononuclear cells (△——△) also responded strongly. Only weak responses were seen with rabbit thymocytes (▲——▲) and mouse peripheral mononuclear cells (□——□).

weakly (Fig. 1). Thymocytes from neonatal rabbits, which responded strongly to Con A (SI = 60·0) (data not shown) showed a weak though significant response to CESA (SI = 10·0, Fig. 1) and to PWM (SI = 12·0) (data not shown). The peak response for CESA was found to be on day 4 or 5.

In the experiments described below cultures were harvested after 84 to 88 h, when Con A and PWM had the highest incorporation rates. The incorporation rates for CESA at this timepoint were about 70 to 80% of the peak response. Antisera to the major protein components of CESA as well as erythrogenic antitoxin did not neutralize the mitogenic activity at all.

Lymphocyte subpopulations responding to CESA. The combined purification steps (nylon wool column and SPA-rosetting using monoclonal antibodies) yielded T cell subpopulations (OKT4 positive and OKT8 positive cells) of high purity. OKT4 cell preparations contained between 0·3 and 1·7% OKT8 positive cells; OKT8 cell preparations contained between 2·9 and 3·5% OKT4 positive cells. Monocyte contamination varied between 0 and 1·8%. The purity of the B cell fraction using SPA-OKT3 rosetting was less satisfactory (about 90% B cells); the contaminating cells were mostly monocytes. In these experiments a dose of 1·0 μg CESA per microculture was used.

Proliferation was highest with unseparated mononuclear cells. Comparing T cell subpopulations, T helper cells (OKT4 positive) incorporated significantly more ^{3}H-thymidine than T suppressor cells (OKT8 positive cells) in the presence of CESA. But when stimulation indices were compared there were no significant differences between the three lymphocyte preparations tested (Fig. 2).

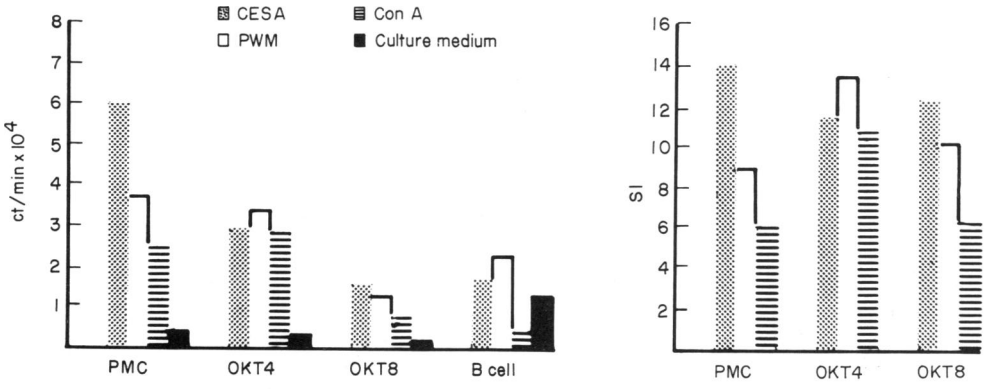

Fig. 2. Response of different cell populations to CESA (1 μg per well). Both absolute ^{3}H thymidine uptake and stimulation indices are shown. OKT4 positive and OKT8 positive cells both responded to CESA, the former better than the latter. B cells did not respond.

The proliferation behaviour to CESA, Con A and PWM differed in the three lymphocyte preparations. CESA had a markedly stronger effect on unseparated mononuclear cells than PWM or Con A. The stimulatory effect of CESA on OKT4 cells was similar to that of the lectins, OKT8 cells responded in general only slightly; in particular the response to Con A was very low (Fig. 2). If the relatively high spontaneous proliferation in the purified B fraction is taken into account then a very weak response to CESA and PWM was seen, which when the low SI (below 2·0) is considered has to be regarded as insignificant (Fig. 2). It should be emphasized that CESA stimulation did not depend on the presence of monocytes.

Role of monocytes. In these experiments the panning method was used for isolation of lymphocyte subpopulations. The cells obtained were nearly pure T cell populations; B cells were absent and monocyte contamination was very low (less than 0·2%). A satisfactory enrichment was only obtained with the OKT4 positive cells (73%), negative selection; the OKT8 positive cells could only be enriched to 50%. The data obtained with these enriched fractions is shown in Fig. 3. A dose of 100 ng CESA per microculture was used. The incorporation rate in the cultures supplemented (5–10%) with monocytes was markedly higher than in the cultures where monocytes had been

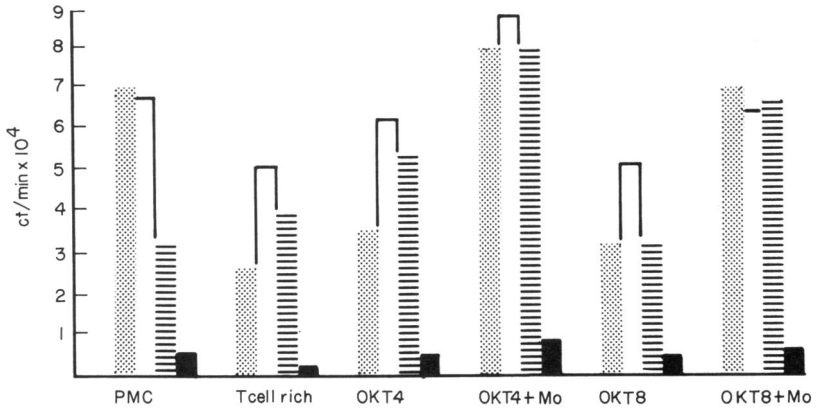

Fig. 3. Response of T cell subpopulations to CESA (100 ng per well). The addition of macrophages enhances the incorporation rates. Key as in Fig. 2.

Table 1. Stimulation index (SI) of various cell populations stimulated with CESA (5 μg per ml), harvested after 84 to 88 h.

Cell	SI
PMC	12·3
T cell rich	13·5
OKT4	8·6
OKT4 + monocytes	10·0
OKT8	8·4
OKT8 + monocytes	11·3

removed. The enhancing effect of monocytes can also be seen in the SIs, although the difference was not as marked as with the incorporation rates (Table 1). Using rabbit thymocytes, no proliferation was obtained with PWM when monocytes had been removed, the responses to Con A and CESA were reduced but still significant (SI = 23·0 resp. 5·0); data not shown.

DISCUSSION

A variety of extracellular products of group A streptococci have been reported to exert stimulatory activity on lymphocytes (Cavaillon, Geoffroy & Alouf, 1979). The mitogenic response is thought to be non-specific (not acquired) since lymphocytes from the umbilical cord and from subjects and animals without evidence of streptococcal infections can respond significantly (Cavaillon, Geoffroy & Alouf, 1979). This is in agreement with our data obtained in newborns (Schneider & Vogt, 1985). However comparing lymphocytes from APSGN patients with those from subjects without evidence of streptococcal infections, markedly higher SIs were observed in the former group when stimulated with CESA (Mosquera *et al.*, 1985), showing that in addition to the mitogenic effect an antigen dependent response can occur.

Some of the streptococcal fractions previously reported to possess mitogenic activity are cationic or contain cationic proteins. This holds true for the EF_2 fraction described by Cavaillon, Geoffroy & Alouf (1979), for blastogen A (Gray 1979) and for erythrogenic toxin B (Knöll *et al.*, 1983).

There are a number of reasons for believing that CESA is not closely related to either blastogen A or erythrogenic toxin. The mitogen activity of CESA could not be neutralized by erythrogenic antitoxin; in fact antibodies reacting with the major protein components of CESA were ineffective in this respect. Blastogen A and streptococcal erythrogenic toxin have a mol. wt of 17,500 D while CESA has a mol. wt of about 30,000 D. Gray (1979) mentioned that reduction of the blastogen A preparation with dithiothreitol resulted in a reduction in the major protein bands and in the generation of a previously absent protein species incident with the blastogenic activity. Gerlach *et al.* (1983) reported that purified erythrogenic toxin B is identical with streptococcal proteinase and the fraction possessing mitogenic activity. CESA was however mitogenically active in a non-reduced state, when it also failed to exhibit proteolytic activity (unpublished data). The EF_2 fraction described by Cavaillon, Geoffroy & Alouf, (1979) has a mol. wt similar to that of CESA, but is otherwise not well characterized, making comparisons difficult.

CESA preparations were mitogenically active at concentrations as low as 0·5 ng/ml and obviously possessed higher mitogenic activity than fractions described by other authors. Crude CESA contains a number of components (all of mol. wt 30,000 D and pI > 8·5); to date we have not been successful in purifying the mitogenic fraction completely (Suzuki, Vogt & Batsford, 1985).

The mitogenic effect of CESA was clear cut and dose dependent. For human and rabbit peripheral leucocytes the maximal response was observed with 5 to 50 μg/ml. Mouse lymphocytes responded only marginally. In kinetic studies the peak response occured later (day 4 to 5) than found for the lectins Con A and PWM but still earlier than the response observed with antigens like *Candida albicans*, Herpes simplex and Cytomegalovirus (day 6 to 8). The mitogenic potency can also be derived from the fact that CESA possesses a strong proliferative activity for lymphocytes in newborns (Schneider & Vogt, 1985). Similar findings in respect of species differences, mitogenic activity, and dose related responses have been reported for blastogen A, EF_2 and erythrogenic toxin B (Gray 1979; Cavaillon, Geoffroy & Aluof, 1979; Barsumian, Schlievert & Watson, 1978). T cells have been also reported to be the target cells for EF_2 (Cavaillin, Geoffroy & Alouf, 1979) and for erythrogenic toxin B (Knöll *et al.*, 1983). When unseparated peripheral leucocytes from adults were stimulated with CESA, in addition to T helper and T suppressor cells, B cells also responded, the predominant proliferating cell type being OKT8 positive cells (Schneider & Vogt, 1985). As shown in this study purified B cells however were not able to respond *in vitro* to CESA, indicating that CESA besides its strong mitogenic quality, can be regarded as a T cell dependent antigen.

The data obtained with pure leucocyte subpopulations revealed two more pieces of information. First, both (OKT4 and OKT8) T subpopulations can respond to CESA and in contrast to the findings with unseparated peripheral leucocytes the predominant cell type responding was the OKT4 positive cell. Second, T cells can respond to CESA without the help of monocytes. This does not mean that monocytes have no effect. The addition of monocytes result in increased incorporation rates, but the same incorporation rates can be reached without the help of monocytes, when the amount of the mitogen (CESA) is increased. Thus monocytes are not absolutely necessary for T cell stimulation by CESA but support the proliferative response and can compensate for higher mitogen doses.

It is still a matter of debate whether T cells are involved in the pathogenesis of poststreptococcal nephritis. The findings reported here are far from being a proof but encourage further investigation in this field.

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