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Characterization of the mitogenic and antigenic stimulatory properties of a purified streptolysin O preparation

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SUMMARY

In vitro transformation of human peripheral blood lymphocytes was induced by a purified group A streptococcal extract rich in streptolysin O (SLO). The stimulation was shown to be largely a general mitogenic effect rather than antigen-selective as a large panel of normal blood donors responded strongly. Further support for a non-specific stimulation was obtained by experiments with cord blood lymphocytes. An antigen-specific response could also be detected as culture supernatants from the stimulation experiments contained immunoglobulins with SLO neutralizing properties. In addition, the total response could partly be inhibited by rabbit heteroantisera against HLA-DR-like antigens supporting the above contention. The stimulatory properties were found to reside in the SLO molecule although results may indicate that the determinants provoking the lymphocyte transformation differ from the determinants possessing the haemolytic properties. Peak response was found on days 4–5. Cell fractionation experiments indicated that only T lymphocytes were stimulated although co-operating B cells and/or monocytes were necessary.

INTRODUCTION

It has been suggested that many of the non-suppurative complications seen during streptococcal infections are due to immunological mechanisms. Cross-reactivity between some streptococcal antigens and tissue antigens is reported (Meyer, 1947; Kaplan, 1963; Zabriskie & Freimer, 1966; Markowitz & Lange, 1964; Husby *et al.*, 1976), whereas other streptococcal membrane constituents bind to lymphocyte surfaces preventing the action of anti-HLA antisera (Hirata & Terasaki, 1970; Hirata *et al.*, 1970). A number of reports have described lymphocyte transforming activities in supernatants from streptococcal cultures (Seravalli & Taranta, 1974; Abe & Alouf, 1976). Thus, it has been claimed that streptolysin S (SLS), a cytotoxic product elaborated by *Streptococcus pyogenes* and some other streptococcal groups, posesses mitogenic properties against human peripheral blood lymphocytes (Hirschhorn *et al.*, 1964). Concerning streptolysin O (SLO), which is another haemolysin obtained from group A streptococcal supernantants, the situation is still unclear. The antigenic properties of SLO are, however, well documented (Wannamaker, 1980).

As other non-specific mitogens are reported to have immunosuppressive effects (Møller, 1970), it would be of interest to establish whether SLO have mitogenic properties or not, and eventually try to relate this activity to the different events described during streptococcal infections.

The present work represents a detailed study of both the mitogenic and antigenic properties of a highly purified SLO preparation. The transformation kinetics as well as the different cell populations involved are described.

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MATERIALS AND METHODS

Lymphoid cell preparations. Mononuclear leucocytes (MNC) were separated from heparinized peripheral blood and umbilical cord blood by flotation on Isopaque-Ficoll gradients (Nyegaard & Co., Norway) (Böyum, 1976). Adherent cells were then removed by incubation in plastic tissue culture flasks. The non-adherent cell population was depleted of T cells by rosetting with neuraminidase-treated sheep erythrocytes (SRBC) (Weiner, Bianco & Nussenzweig, 1973) with subsequent gradient centrifugation on Isopaque-Ficoll. B cell enriched lymphocytes were collected from the interphase whereas the purified T lymphocytes were treated with autologous serum to lyse away the SRBC (Lea *et al.*, 1979). Adherent cells were recovered from the culture flasks by incubating with EDTA solution on ice followed by vigorous pipetting (Bergholtz & Thorsby, 1978). B lymphocytes were scored by direct immunofluorescence technique (Frøland, Natvig & Stavem, 1972), while T lymphocytes were enumerated by using a common rossette assay (Frøland & Natvig, 1973). The content of monocytes in the adherent cell fractions were estimated by morphological criteria together with staining for peroxidase (Kaplow, 1965) and α-naphtyl acetate esterase activity (Horwitz *et al.*, 1977). All cell populations were thoroughly washed thrice with HBSS (Flow Laboratories, Scotland) prior to cultivation.

Bacterial antigens and mitogens. Group A streptococci strain S84 were used after inactivation by heat and formaldehyde fixation. PHA (Wellcome Research Laboratories) was used in dilution of 1:50, PWM (GIBCO, Scotland) 1:4, concanavalin A (Sigma Chemical Co., Missouri, USA) 125 μg/ml, PPD (Statens Seruminstitut, Copenhagen, Denmark) 1 mg/ml and SLO 100 μg/ml. All mitogens were diluted in HBSS.

Cell cultures. Triplicate cell cultures were set up in flat bottomed Costar 3596 micro-tissue culture plates, each culture containing 50×10^4 lymphoid cells and 20 μl antigen or mitogen in a total volume of 120 μl with RPMI 1640 containing 20% fetal calf serum (FCS) and antibiotics. Control cultures did not contain antigen or mitogen. The mitogen stimulated cultures were harvested on day 3, while PPD stimulation was terminated after 6 days. The duration of SLO stimulation was as indicated in each experiment. Twenty-four hours prior to harvesting 1 μCi ^{3}H-thymidine (Sp. act. 2 Ci/mmol, Radiochemical Centre, Amersham, England) was added to each culture. Harvesting was performed with distilled water onto glass fibre filters using a semi-automatic harvesting machine (Skatron, Lierbyen, Norway), and radioactivity measured by liquid scintillation counting.

Isolation of monoclonal proteins. Monoclonal IgG components possessing SLO neutralizing activity were isolated from two patients, SO and EA, with monoclonal gammopathy. The EA protein was eluted from DEAE–cellulose (Whatman DE-52) with 0·015 M sodium phosphate buffer, pH 7·6. The SO component was isolated from QAE Sephadex (QAE-A50, Pharmacia, Sweden) with 10 mM Tris-HCl pH 8·13 using a linear gradient of NaCl from 0–0·3 M in the above buffer.

Isolation of F(ab′)$_2$ fragments. Monoclonal proteins and IgG fractions of rabbit antisera were pepsin digested and F(ab′)$_2$ fragments isolated by gel filtration on calibrated G-200 columns under neutral conditions as described elsewhere (Michaelsen & Natvig, 1974).

Measurement of SLO neutralizing activity. The anti-SLO activity was determined by a standard procedure at the Department of Bacteriology, National Institute of Public Health, Oslo. The anti-SLO activities in the patients' sera were; EA 166,000 iu/ml, and SO, 49,200 iu/ml.

Antisera. Antisera were raised in rabbits by primary immunization with the antigens emulsified in Freund's complete adjuvant and at least five booster injections. As antigens were used F(ab′)$_2$ fragments of the various monoclonal immunoglobulins. The anti-idiotype antisera were rendered idiotype specific by absorption on immunosorbent columns containing normal human IgG (Kabi AB, Sweden) or normal human AB serum as previously described (Lea *et al.*, 1979). The idiotype specificity of the antisera were ascertained in a sensitive ELISA system (Michaelsen *et al.*, 1980). As anti-HLA-DR antibodies was used an IgG fraction of anti-p 23,30 antiserum (kindly provided by Dr J. Strominger).

Isolation of SLO. SLO was isolated from supernatants of streptococcal cultures by ammonium sulphate precipitation at 75% saturation. The precipitate was dissolved in a small volume of water,

dialysed against 0·05 M Tris-HCl buffer, pH 8·5 and further purified on DEAE–cellulose ion exchanger (Whatman DE-52). The main haemolytic activity was eluted by a linear salt gradient (0–0·3 M NaCl) in the same buffer. Fractions containing haemolytic activity were pooled, lyophilized, and further purified by gel filtration on Sephadex G-200. The haemolytic activity was eluted corresponding to a mol. wt of approximately 70,000 daltons.

RESULTS

Stimulatory properties of the SLO preparation

The stimulation of peripheral blood MNC obtained with the purified SLO preparation in comparison with some commonly used mitogens and antigens is depicted in Fig. 1. Twenty normal blood donors are included in this panel showing a SLO response by thymidine incorporation in between the Con A and PWM responses.

Stimulation kinetics

To optimize culture conditions, the time-dependent and dose–response relationship were investigated. Peak response was found on day 4–5 with an SLO concentration of approximately 100 μg/ml (Fig. 2). As SLO is shown to contain very few aromatic amino acids, this concentration has been calculated after amino acid analysis of the purified preparation.

Evidence for the blastogenic activity of SLO

To reveal whether SLO or some other contaminating substances were responsible for the blastogenic activity, the SLO preparation was prior to stimulation incubated with $F(ab')_2$ fragments of the SO monoclonal IgG possessing SLO neutralizing activity. This treatment almost completely abolished the mitogenic activity, whereas no effect was seen on PHA, PWM or Con A responses (Fig. 3). To ascertain that this inhibition was due to SLO being specifically bound by the SLO neutralizing antibodies, the fragments were incubated with $F(ab')_2$ fragments of rabbit anti-idiotype

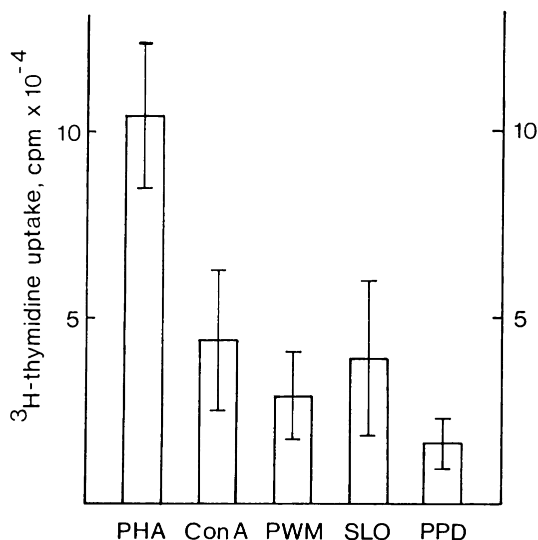

Fig. 1. Stimulatory properties of SLO together with some commonly used mitogens and antigens. SLO stimulated cultures were harvested on day 5, PPD cultures on day 6 while the others were terminated on day 3. Stimulation was measured by ^{3}H-thymidine incorporation and results expressed as arithmetic means and ranges of 20 normal blood donors.

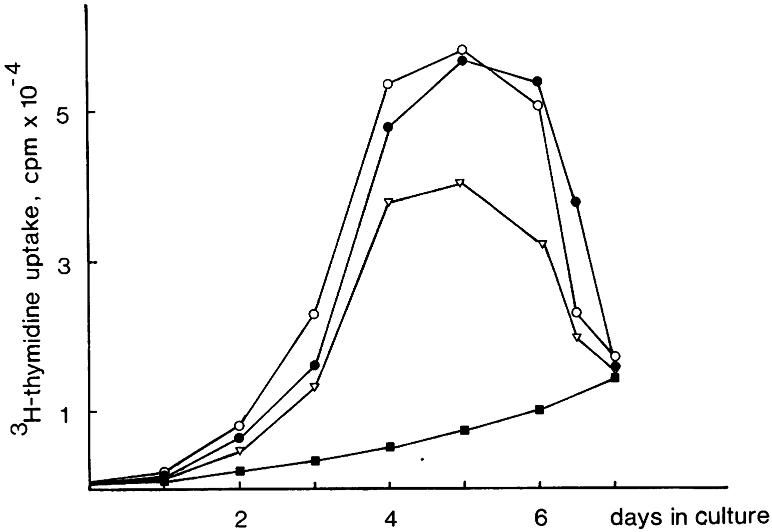

Fig. 2. Stimulation kinetics of the SLO response. Peripheral blood MNC from 3 different donors were tested with the following concentrations of SLO: (▽) 25 μg/ml, (○) 100 μg/ml, (●) 200 μg/ml and (■) medium control. Results are expressed as arithmetic means.

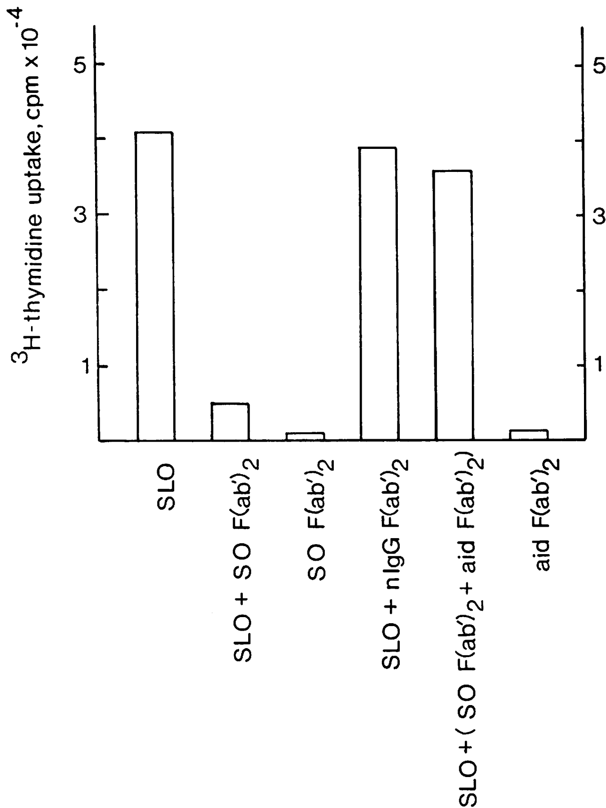

Fig. 3. The effect of addition of F(ab')2 fragments of SO monoclonal component with SLO neutralizing properties and normal human IgG (nIgG) on SLO-induced responses. The effect of F(ab')2 fragments of anti-idiotypic rabbit anti-SO antibodies (aid) is also shown. The results represent means of three different experiments.

Table 1. PHA- and SLO-induced responses in MNC from three cord blood donors

	^{3}H-thymidine incorporation (c.p.m.)	
	SLO 100 μg/ml	PHA
Donor 1	23,687	58,232
Donor 2	11,337	39,600
Donor 3	4,532	18,458

antibodies against the SO protein before adding SLO and lymphocytes. This treatment should block the antigen binding site of the anti-SLO antibodies and thus permit SLO to exert its blastogenic effect. Fig. 3 shows that the stimulatory capacity of SLO was fully restored.

Assessment of the nature of the blastogenic response

In an attempt to reveal whether the blastogenic response was caused by mitogenic or by antigenic stimulation, experiments were performed with cord blood MNC from three different donors. The results showed varying but very significant thymidine incorporation in all three cases (Table 1) supporting our view that the proliferative response caused by SLO was due primarily to mitogenic transformation of the lymphocytes.

However, culture supernatants harvested on day 5 from peripheral blood MNC stimulated with SLO regularly contained significant SLO neutralizing capacity. These results also indicated an antigen-specific part of the overall response.

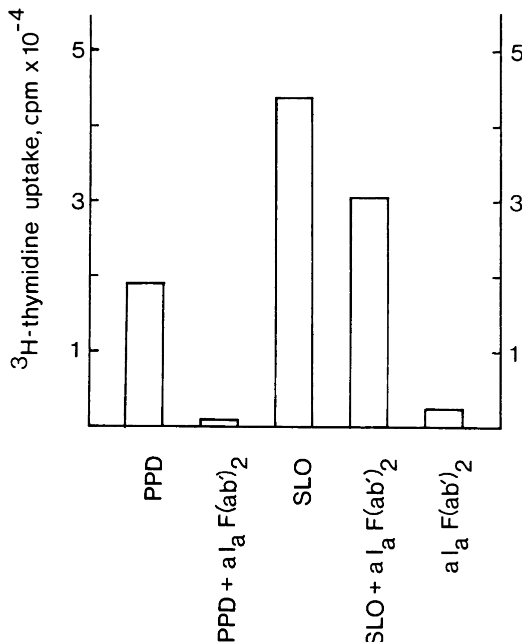

Fig. 4. The effect of anti-HLA-DR antibodies (aI$_a$) on PPD- and SLO-induced lymphoproliferation. Results represent means of five different experiments.

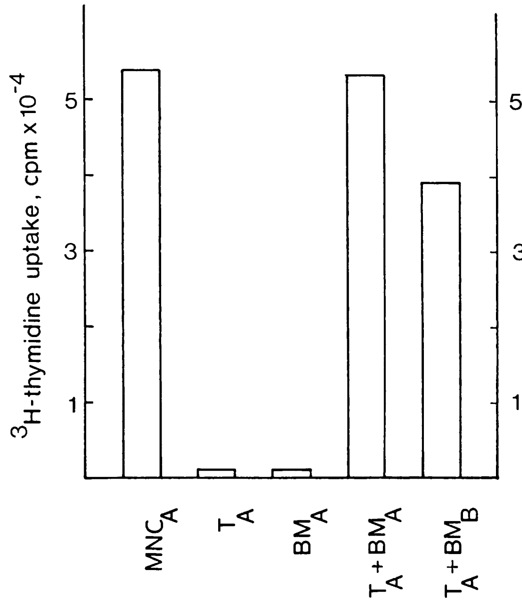

Fig. 5. Co-cultivation experiments showing the effect of allogeneic adherent cells on the SLO induced response. T depleted MNC from donor B were added to purified T lymphocytes from donor A in numbers corresponding to what was found in peripheral blood. The results represent means of three different experiments.

To test this possibility further, blocking experiments were carried out with the addition of anti-HLA-DR-like antibodies during cultivation. This resulted in a diminished response both measured by thymidine incorporation (Fig. 4) and by a reduced inhibitory capacity of the culture supernatant in the standard SLO assay (data not shown). As controls were used cultures stimulated with PHA, Con A and PWM. Addition of anti-HLA-DR-like antibodies in these systems had no measurable effect on cell proliferation as assayed by thymidine incorporation.

Co-cultivation experiments using allogeneic adherent cells showed a strong SLO proliferative response, although significantly reduced in comparison with the autologous control cultures (Fig. 5).

Table 2. Purity of the various cell populations used

	SIg^{+}*	E-RFc†	Pox^{+}‡
MNC	14	63	16
B lymphocyte enriched population	>96	<2	1
Plastic adherent cells	26	1	68
T lymphocyte enriched population	<1	>90	<1

* Cells with membrane-bound immunoglobulin.
† Cell forming rosettes with sheep erythrocytes.
‡ Cells with peroxidase activity in plasma.

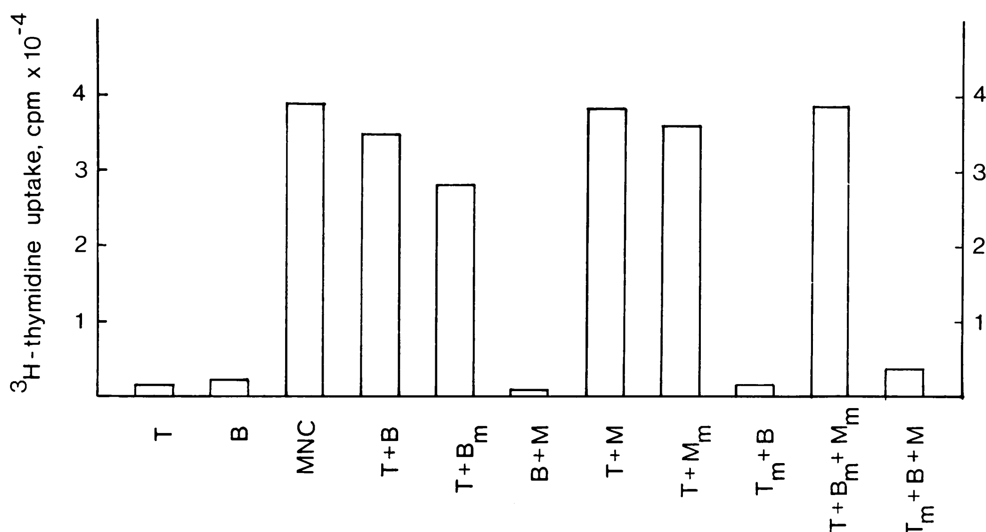

Fig. 6. These experiments show SLO-induced responses in peripheral blood MNC, isolated cell populations and admixtures thereof, together with mitomycin C (m)-treatment of the various cell populations. T, B and M denote T cell enriched, B cell enriched and monocyte enriched cell populations respectively.

Cell populations involved in the SLO response

To assess which cell populations were responding, cell fractionation experiments were carried out. The purity of the various cell fractions is shown in Table 2. Neither isolated T nor B cells or monocytes responded, but adding B cells or monocytes to T cell populations in proportions corresponding to what is normally found in peripheral blood, resulted in as high a response as obtained with peripheral blood MNC (Fig. 6). By combining mitomycin C inactivation of the cell populations with the above type of experiments, results indicated that T lymphocytes were responsible for the proliferative response. However, for T cell activation to occur, the presence of B cells and/or monocytes were necessary. Inactivation of the B lymphocytes and monocytes by mitomycin C treatment seemed to have very little impact on the overall response (Fig. 6).

DISCUSSION

The present study provide evidence for streptolysin O (SLO) posessing strong mitogenic properties. This conclusion rests on a series of experiments with a highly purified SLO preparation derived from strain S84 culture supernatants. Firstly, stimulation with SLO resulted in markedly strong responses with peripheral blood MNC from all donors in a large panel. Measured by thymidine incorporation the lymphoproliferation was comparable to Con A responses. However, the peak response was seen on day 4–5 which is later than found for strong mitogens like PHA, Con A and PWM, but still earlier than the responses provoked by antigens like PPD, candida, herpes virus etc. This late peak response has, however, been reported for other mitogens and is possibly a function of fewer receptors or weaker interaction with the receptors on the cells binding the mitogen. Another argument for the mitogenic properties of SLO comes from experiments with cord blood MCN, where we found varying but significant responses in the three donors tested. Furthermore, experiments with isolated monoclonal proteins having SLO neutralizing properties provide undisputable evidence for the mitogenic factor being part of the molecule having the haemolytic activity. We cannot conclude whether these two activities reside in the same determinants on the

SLO molecule since blocking of the haemolytic properties by anti-SLO antibodies might lead to steric hindrance of the mitogenic determinant as well. It might be worth mentioning that the mitogenic effect of the preparation decreases during storage at −20°C, while the haemolytic activity is essentially unchanged (unpublished observations).

Cell fractionation experiments clearly demonstrated that active T cells were an absolute pre-requisite for stimulation to occur. No transformation could be detected when mitomycin C treated T cells were added to a mixture of B cells and monocytes, thus excluding B lymphocytes or monocytes as the target cell for the mitogenic effect of SLO. T cell populations containing less than 1% surface Ig positive and less than 1% peroxidase positive cells could not be stimulated, and B cells or monocytes had to be added to restore transformation. From our experiments it is hard to say whether B lymphocytes or monocytes are responsible for the co-operative effect since both cell populations to a certain degree seem to be cross-contaminated (Table 1). However, since the number of monocytes in the B cell population is relatively low it could be that the T cell blastogeneic effect is B cell dependent. Still, we know from other mitogen transformation studies, that the number of monocytes necessary for providing adequate help seem to be very low (Taniguchi *et al.*, 1977). Furthermore, mitomycin C treatment of the accessory cells had very little impact on the overall response. Experiments trying to compensate for co-operating cell requirements by addition of varying amounts of β-mercaptoethanol were all unsuccessful (unpublished observations).

The antigenic stimulatory activity of SLO was investigated by various assay methods. Small but significant amounts of antibodies with SLO neutralizing properties could be detected in culture supernatants harvested on day 5 by the standard assay. It is well established that for an antigen-specific T cell response to take place there must be compatibility at the HLA-D/DR level between adherent accessory cells and the antigen-specific T lymphocytes (Bergholtz & Thorsby, 1977). Experiments with anti-HLA-DR-like antibodies inhibiting antigen-specific T cell proliferation support the notion that HLA-D/DR antigens are directly involved in the macrophage presentation of soluble antigens to T lymphocytes (Bergholtz & Thorsby, 1978). On the other hand, it has been suggested that the potentiating effect of adherent cells on various mitogen-induced T cell and T cell-dependent proliferative reactions is mediated by soluble factors (Rosenstreich & Mizel, 1978). Thus, HLA-D/DR disparate adherent cells may function as accessory cells during T cell responses triggered by polyclonal activators making it possible to distinguish between mitogen and antigen-specific T cell responses. Our experiments with anti-HLA-DR-like antibodies and allogeneic adherent cells during the SLO response confirm this notion. The anti-HLA-DR-like antibodies almost ablated PPD-induced responses while PHA and Con A responses were virtually unaffected (data not shown). The SLO response, on the other hand, were reduced by approximately 30%. The same was found in the studies with allogeneic adherent cells. The experiments were conducted under conditions minimizing cell-to-cell contact. Also, harvesting on day 4 reduced the problems with the ongoing mixed lymphocyte reaction. These results thus support the view that the overall blastogenic response induced by this purified SLO preparation consists of a minor antigen-specific response together with a strong mitogenic response.

The antigen specific part of the total response is also reflected in proliferating B lymphocytes secreting SLO neutralizing antibodies into the culture supernatants. This antibody production was apparently depending on specific T helper cells as indicated by its sensitivity to inhibition by anti-HLA-DR-like antibodies.

As the overall blastogenic response was completely inhibited by the monoclonal SO component, both the antigen-specific T and B lymphocytes possibly react with the same determinant on the SLO molecule. In experimental animals, it has been shown that antibodies to protein antigens do not inhibit T cell proliferative responses to that antigen (Ellner & Rosenthal, 1975; Ellner, Lipsky & Rosenthal, 1977) suggesting that the antigenic determinants recognized by the T cell are either inaccessible to the antibody or differ from those recognized by antibodies against native antigen. Our results indicate that this proposal is not generally valid. In fact we have earlier published that anti-idiotypic antibodies raised against the monoclonal SO protein detect cross-reacting determinants on the patient's own T and B lymphocytes (Lea *et al.*, 1979) thus supporting the above contention.

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