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The inhibitory effects of mycobacterial lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation

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SUMMARY

Lipoarabinomannan from *Mycobacterium tuberculosis* was able to inhibit antigen induced T cell proliferation of human $CD4^+$ T cell clones specific for influenza virus. The inhibitory effect was also present when peripheral human T cells were stimulated with crude mycobacterial antigen extracts. Non-specific T cell stimulation, i.e. IL-2, PHA and anti-CD3 antibodies coupled to beads, was not affected. The inhibitory property was also found when arabinomannan and arabinogalactan of mycobacterial origin were tested but not with other unrelated polysaccharides used as controls. The effect appears to be related to the processing of the antigen by the antigen-presenting cells, since it was evident when T cell clones were stimulated with whole virus, whereas stimulation with a synthetic peptide containing the relevant epitope was not inhibitable.

Keywords lipoarabinomanannan mycobacteria T cell proliferation

INTRODUCTION

Mycobacterial polysaccharides have been the subject of extensive analysis over a period of many years from the point of view of their chemical structure and their interaction with humoral and cellular components of the immune system. It has recently been shown that, in its native form, the arabinomannan (AM) polymer of mycobacteria possesses a lipid tail, a phosphatidyl inositol component and complex succinyl and lactyl acylations in addition to the previously described carbohydrate backbone (Hunter, Gaylord & Brennan, 1986). Previous studies on AM included a preliminary alkaline hydrolysis step which resulted in removal of the acyl substituents from the purified material, as demonstrated by Weber & Gray (1979). While antibodies to the arabinomannan backbone clearly occur (Misaki, Azuma & Yamamura, 1977), the immunodominant sites on lipoarabinomannan (LAM) as defined by monoclonal antibodies raised against *Mycobacterium leprae* apparently involve the α linked-D-arabinofuranosyde residues (Gaylord *et al.*, 1987).

Mycobacterial arabinomannan has previously been shown to cause an inhibitory effect when added to antigen dependent lymphoproliferation assays (Ellner & Daniel, 1979; Ellner & Spagnuolo, 1979). The degree of acylation of the AM preparation used in these studies was not tested but its chemical similarity to the alkaline hydrolysed form was noted. In view of the importance of the acyl modifications for antibody recognition, it is appropriate to reassess the interaction of AM with the cellular immune system using intact LAM preparations. Kaplan *et al.* (1987) have recently studied the effect of LAM on the lymphoproliferative response of leprosy patients and suggest that one effect of LAM may be to induce suppressor T cells.

The aims of this study are to investigate the modulatory effects of LAM and other polysaccharides on the interleukin-2 (IL-2) and antigen dependent proliferation of human peripheral blood mononuclear cells and cloned T lymphocytes. The effect on T cell proliferation was found with both systems but only when antigen was given a unprocessed form, suggesting that the antigen presenting cells and not the T cells are the targets for the modulatory effect of the polysaccharides.

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MATERIALS AND METHODS

Antigens

Formalin inactivated influenza virus A/Texas/1/77 × 49(H_3N_2) was the kind gift of Dr. A.S. Rosenthal, Merck, Sharp and Dohme, Rahway NJ. Immunochemically purified haemagglutinin (HA, A/Bangkok/1/79;H_3N_2) was kindly provided by Dr. R.S. Webster, St. Jude Children's Research Hospital, Memphis, TN. The synthetic peptide (p14) amino acid sequence 306–319 of the HA-1 molecule of influenza virus haemagglutinin was obtained from Peninsula Laboratories, Cambridge. The soluble extracts of *Mycobacterium tuberculosis* (MTSE) were prepared

as described elsewhere (Young *et al.*, 1986). Purified protein derivative (PPD) was obtained from Evans Medical Ltd., Greenford, Middlesex. Lipoarabinomannans (LAM) from two different sources were used: one was a generous gift from Dr. P. Brennan, the other was prepared from irradiated *Mycobacterium tuberculosis* (H37Rv) cells using the method adopted from Hunter *et al.* (1986). Briefly, the cells were delipidated using chloroform: methanol 2:1, then refluxed in 50% (w/v) ethanol. Reflux was carried out for a total of 6 h with three changes of solvent. The ethanol extract was concentrated, treated with RNA-ase and DNA-ase (Boehringer) then extracted twice with phenol at 80°C. The water soluble layer was then extracted with chloroform methanol (2:1), dialysed, freeze-dried, and redissolved in tris buffer (10 mM pH 7·4) containing 1% (w/v) Triton × 100 (BDH Chemicals UK). The sample was loaded onto a DEAE Sephacel (Pharmacia UK) column (24 × 0·9cm) eluted with the same buffer. After loading and washing with two column volumes of buffer, the column was eluted with a gradient of 0–1 M NaCl in the starting buffer, and 3 ml fractions were collected. Fifty-microlitre samples from each fraction were assayed by solid phase ELISA using ML34 monoclonal antibody as primary antibody and horseradish peroxidase linked rabbit anti-mouse IgM as secondary antibody. The positive fractions were pooled, dialysed, and freeze-dried. In order to remove the trace of Triton in the sample, it was redissolved in a small volume of water and the carbohydrate was precipitated with ethanol using ammonium acetate as precipitant. This procedure was repeated twice and the resulting precipitate was tested for LAM content and purity by various methods. SDS-PAGE was performed on a Hoeffer 'Mighty Small' apparatus, using 12% acrylamide gels. After electrophoresis the gel was fixed in 10% (w/v) trichloroacetic acid, then treated with 1% (w/v) periodic acid in 3% (w/v) acetic acid for 1 h. After extensive washing with distilled water the gel was immersed in Schiffs reagent (BDH Chemicals UK); LAM showed as a broad band located at a molecular weight of 38–40 kD. Western blot analysis, using a Jancos semi-dry blotter and Hybond C (Amersham UK) nitrocellulose, showed that this band stained with ML34. Samples of LAM were also assayed by gas-liquid chromatography (GLC). Alditol acetate derivatives were prepared according to Gunner, Jones & Perry, (1961) and fractionated using a 5% CP-Sil 88 chromopack column run at a temperature gradient of 180–220°C on a Pye Unicam Chromatograph equipped with a flame ionisation detector. Carbohydrate standards (Sigma Chemical Co) were used to calibrate the column using 2-deoxy glucose as internal standard. Analysis showed a 63% carbohydrate content and a molar ratio of arabinose to mannose of 2·5:1. The GLC analysis indicated the presence of glucose (~1%) and galactose (~2%) in the preparation. *Mycobacterium tuberculosis* (AM) and arabinogalactan were provided by Dr. P. Draper, NIMR, Mill Hill, London. Phytohaemagglutinin (PHA) was purchased from Wellcome Research Laboratories. Larch wood Arabino galactan was purchased from Sigma Chemical Co. Levan (from *Corynebacterium levaniformis*) and Dextran B1355 (from *Leuconostoc mesenteroides*) have been described before (Moreno, Courtenay & Howard, 1976; Moreno, Hale & Ivanyi, 1977) Ficoll was purchased from Pharmacia UK. Anti-CD3 antibody coupled to sepharose 4B (CD3-sepharose) was kindly provided by Dr. M. Owen, ICRF Tumour Immunology Unit, University College, London.

Table 1. Effect of LAM on the proliferative response of PBMC to antigen and mitogen

	LAM (μg/ml)			
Antigen*	0	0·3	3	30
Medium†	2202(46)	984(40)	2352(17)	471(11)
MTSE	32980(8)	38349(21)	39554(14)	18850(3)
PPD	37224(23)	53987(25)	31960(25)	8114(25)
PHA	77164(5)	85831(4)	80483(7)	82595(1)

Proliferation as correlated with ^{3}H-TdR incorporation was determined at 3 and 6 days for mitogen and antigen respectively. The results are expressed as mean ct/min ± s.e.m % of triplicate cultures. Values underlined are statistically different from controls with a confidence value > 99%.

* Antigen (MTSE, 1 μg/ml; PPD, 1 μg/ml) or mitogen (PHA, 1/200 v.v) were added at the initiation of the cultures alone or together with LAM.

† Control responses of PBMC in medium and to LAM alone without added antigen or mitogen.

Table 2. Effect of various lipopolysaccharides on the proliferative response of PBMC to PPD

Antigen	Polysaccharide concentration (μg/ml)	Response (ct/min ± % sem)	% inhibition of PPD response
PPD alone	—	75837 ± 10	0
LAM	3	26284 ± 10	65 ± 1
(M. tuberculosis)	30	782 ± 43	99 ± 6
AM	3	34149 ± 47	55 ± 1
(M. tuberculosis)	30	20279 ± 36	73 ± 1
D-Arabino-D-galactan	3	21576 ± 49	72 ± 1
(M. tuberculosis)	30	1094 ± 52	99 ± 5
L-Arabino-D-galactan	3	69565 ± 47	13 ± 1
(Larchwood)	30	65663 ± 53	8 ± 1
Levan	3	73012 ± 21	4 ± 1
(Corynebacterium levaniformis)	30	72711 ± 45	4 ± 2

Data underlined differ significantly for controls with a confidence value > 99%.

Lymphocyte preparation

Mononuclear leucocytes (PBMC) were isolated from peripheral blood by centrifugation on Ficoll-Hypaque density gradients and resuspended in RPMI-1640 supplemented with 2 mM L-glutamine, penicillin/streptomycin at 100 iu/100μg/ml and 10% pooled A^+ serum.

Isolation of antigen-specific T lymphocyte clones

Human influenza virus reactive T cell clones were isolated and characterized as described previously (Lamb *et al.*, 1982 a,b).

Table 3. Effect of LAM on antigen and IL-2 dependent proliferation of cloned T lymphocytes (ct/min)

T cell clone	Stimulus	LAM μg/ml 0	0·3	3·0	30
HA1.7	medium	148(3)	122(6)	101(6)	84(1)
+	A/Texas	70690(4)	56913(31)	36160(38)	27438(11)
+	IL-2	11036(6)	9374(9)	9357(6)	10747(5)
FL1-37	medium	91(11)	61(9)	63(17)	95(28)
+	A/Texas	78491(1)	79480(1)	57276(1)	34448(1)
+	IL-2	26416(2)	31248(5)	33484(3)	25446(4)

T cells of clones HA1.7 or FL1-37 (10^4/well) were stimulated with A/Texas in the presence of irradiated histocompatible PBMC ($2{\cdot}5 \times 10^4$/well) or with IL-2 alone. LAM was added at the initiation of cultures. Proliferation was determined at 3 days as described in the legend to Table 1. Figures underlined differ significantly from controls (confidence level >99%).

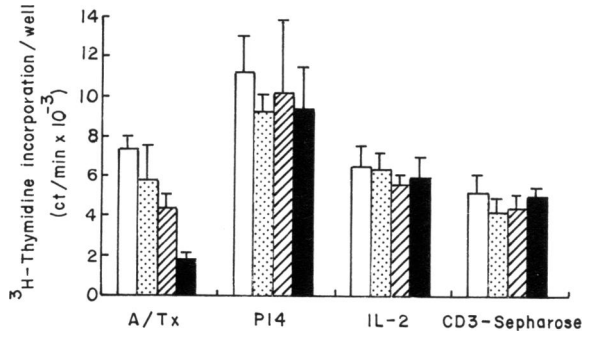

Fig. 1. Inhibition of influenza virus-induced but not peptide-induced proliferation of cloned helper T cells by LAM. T cells of clone HA1.7 were stimulated with A/Texas, p14 or CD3-Sepharose together with irradiated histocompatible PBMC or with IL-2 alone in the presence or absence of LAM. Proliferation was determined at 3 days as described in the legend to Table 1. Data expressed as mean ± standard error. LAM concentrations: □ 0 μg/ml, ▨ 0·3 μg/ml; ■ 3 μg/ml; ■ 30 μg/ml.

Briefly, activated T cells from a 6 day culture of PBMC ($1{\cdot}5 \times 10^5$/ml) stimulated with A/Texas/1/77 (5 HAU/ml) or HA (0·1 μg/ml) were cloned by limiting dilution (one cell every third well) in Microtest II trays (Falcon) with irradiated autologous PBMC $(10)^4$/well), specific antigen and 20% interleukin 2 (IL-2). Clones were expanded with fresh IL-2 every 3–4 days and irradiated PBMC and influenza virus every 7 days. Before use in proliferation assays the T cells were rested 6–8 days after the addition of filler cells. In the experiments reported here the following influenza virus immune clones were used: FLI-37 specific for the matrix protein of influenza A virus (Lamb *et al.*, 1982c) and HA1.7 for the carboxyl terminus of the HA-1 molecule (Lamb *et al.*, 1982b). However, both clones responded to the intact virus.

Proliferation assays

Cloned T cells ($2{\cdot}5 \times 10^4$/ml) were cultured with antigen (A/Texas/1/77, 5 HAU/ml; p14, 0.3 μg/ml; CD3-sepharose, 500 beads/well) in the presence of irradiated histocompatible PBMC ($1{\cdot}25 \times 10^5$/ml) or with IL-2 alone in microtitre trays in complete medium containing 10% A^+ serum. Following 60 h incubation, the cultures were pulsed with 1·0 μCi of tritiated methyl thymidine (^{3}H-TdR; Radiochemicals Inc, Amersham) then harvested onto glass fibre filters. Proliferation as correlated with ^{3}H-TdR incorporation was measured by liquid scintillation spectroscopy. The duration of culture of PBMC with antigen (MTSE) or mitogen (PHA) was 6 and 3 days respectively. The results are expressed as mean counts per minute (ct/min) ± % error of the mean for triplicate cultures.

RESULTS

Addition of LAM inhibits proliferative response of PBMC to antigen

LAM was added at various concentrations to cultures of PBMC alone or those stimulated with MTSE, PPD or PHA (Table 1). While LAM itself induced no marked proliferation as compared to the medium control, its presence at 30 μg/ml resulted in a decrease of 43% in the response to MTSE and 78% to PPD (Table 1). In contrast LAM had no inhibitory effects at any of the concentrations tested on PHA induced proliferation. Table 2 shows the effects of LAM and of various polysaccharides on the response of human PBMC to PPD. All the mycobacterial extracts inhibit the response. The intact LAM completely inhibited the PPD response when added to the culture at a concentration of 30 μg/ml, D-arabino galactan showed similar effects and AM decreased the response by 75%. However, arabinogalactan (containing L-arabinose from larch wood) and levan had negligible effects on the PPD response in these assays.

Effect of LAM on antigen-dependent and IL-2 dependent proliferation of cloned T lymphocytes

In order to determine the specificity of the inhibition mediated by LAM (Table 1), its effect on the proliferative response of T cell clones reactive with influenza viral antigens and IL-2 was investigated. The addition of LAM at 30 μg/ml and, to a lesser extent, 3 μg/ml caused marked inhibition of the response of both T cell clones HA1.7 and TLC37 to influenza virus (Table 3). In contrast, LAM had no inhibitory effects on the IL-2 induced proliferation of the T cell clones.

Failure of LAM, AM and AG to inhibit presentation of a peptide antigen

The inability of LAM to inhibit the IL-2 dependent proliferation of the T cell clones suggested that its effects were mediated at the level of the antigen presenting cell population rather than directly on the T cells. Therefore, the effect of LAM on T cell activation by a 'preprocessed' peptide antigen was investigated. The epitope recognized by T cell clone HA1.7 is located on a 14 amino acid peptide (p14) and the comparative effect of LAM on assays with the peptide antigen and with the intact influenza virus was tested (Fig. 1). While LAM at concentrations of 3 and 30 μg/ml inhibited the proliferative response to intact virus, no marked effect on the response to the peptide was observed. Similarly proliferation of the T cells induced by IL-2 or anti-CD-3 sepharose in the absence of accessory cells was not inhibited (Fig. 1). Arabinomannan and arabinogalactan behaved in a similar fashion (Fig. 2). AG showed a small

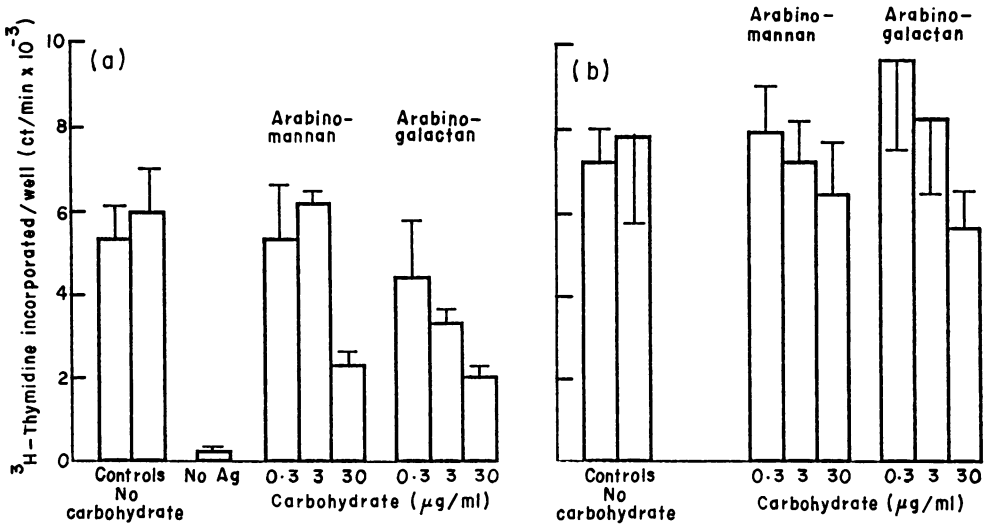

Fig. 2. Inhibitory effect of arabinomannan and arabinogalactan upon the proliferative response of T-cell clone HA1.7 to A.Texas virus (a) and P14 peptide (b). Conditions are identical to those described for Table 1. Data are expressed as for Fig. 1.

increase at 0·3 μg/ml and a modest decrease at 30 μg/ml but neither of these effects were significantly different from control values.

Effect of other polysaccharides on T cell clone proliferation

Just as for PBL stimulation, other polysaccharides used as controls, i.e. levan, Dextran B1355 and Ficoll, failed to inhibit the proliferation of T cell clone HA1.7 at the same concentrations used for the mycobacterial polysaccharides. Moreover, levan and Dextran showed a modest but clear dose-dependent stimulatory effect when the clone was challenged with the peptide (p14) (data not shown).

DISCUSSION

The intact and fully antigenic lipoarabinomannan of *M.tuberculosis* (LAM) shows a comparable activity to previous mycobacterial 'arabinomannan' preparations in causing an inhibition of T cell proliferation during *in vitro* assays with PBMC from normal and tuberculosis patients (Ellner & Daniel, 1979). This result is also in agreement with the findings of Kaplan *et al.*, (1987) on the effect of LAM on *in vitro* assays with PBL's from leprosy patients. Thus, the activity of this molecule in assays of cellular immunity is apparently a function of the arabinomannan backbone rather than of the complex acyl modifications present on the native structure.

The ability of LAM to inhibit the proliferative response of cloned helper T cells reactive with influenza virus demonstrates that the inhibition is non-specific and not restricted solely to the T cell response to mycobacterial antigens. The failure of LAM to inhibit the PHA response of PBMC or the activation of helper T cell without accessory cells suggests that it may modulate specific immune responses at the level of the accessory cell. Although the activation of clone HA1.7 by the appropriate peptide (p14) requires the presence of accessory cells, no further processing of the antigen appears to be necessary (Londei *et al.*, 1984). Indeed as LAM inhibited the presentation of intact influenza virus but not p14, this implies that 'antigen processing' (Unanue, 1984) is one target. Additionally it has been suggested that mycobacterial lipopolysaccharides inhibit *in vitro* antigen and mitogen induced T cell proliferation by the release of prostaglandins (Ellner & Spagnuolo, 1979). However, the failure of LAM to inhibit the response of either PBMC to PHA or cloned TH cells to peptide antigen in the presence of accessory cells suggests that prostaglandin release is not the only mechanism of inhibition and that the effect is not just the downregulation of MHC Class II. The lack of inhibition on CD-3 activated T cell clones suggests that inhibition is not at the level of the T cell. Since our experiments investigated the effect of LAM on only helper T cell clones we are unable to ascertain the effect of LAM on any other T cell subset. Recently it has been reported that LAM can induce $CD8^+$ T cells capable of suppressing the PPD response of both tuberculoid and lepromatous leprosy patients (Kaplan *et al.*, 1987).

A considerable amount of research on suppressive effects induced by mycobacterial antigens has been carried out with particular emphasis on the situation in lepromatous leprosy (reviewed by Gaylord & Brennan, 1987). Most of the experiments addressing this question have involved highly complex interactions between heterogeneous cell populations and unfractionated antigen mixtures, and the results have often been difficult to interpret since they represent a variety of different effects superimposed on each other. This effect might bias results obtained with assays using partially purified antigen for T cell proliferation, such as celloblot analysis of T cell repertoire (Young & Lamb, 1986) where some of the protein antigens overlap with LAM in the acrylamide gel. It is also important to point out that the experiments presented here demonstrate the inhibitory effect of LAM and other polysaccharides at the effector level of T cell proliferation and say nothing about the potential influence upon the inductive phase of the response. In order to understand the nature and number of the inhibitory effects of mycobacterial antigen mixtures it is necessary to fractionate different components and to analyse their effects independently. These effects can be separated into several

categories such as the negative modulation of monocyte function, inhibition of lymphocyte function mediated by monocyte-derived factors, and inhibition mediated by antigen-specific suppressor T cells. The present study indicates that the inhibitory activity mediated by LAM belongs in the first of these categories, that of inhibition of monocyte function, and need not implicate CD8 (TS) in every case since the mediator of inhibition was TH clone+viral PBL+virus inhibition with LAM. The approach described here using a well-defined clonal T cell system will be of use in analysis of the specificity and mechanism of action of inhibitors or suppressive components of mycobacteria.

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