Clin. exp. Immunol. (1977) 29, 176–180.

BRIEF COMMUNICATION

Effect of *A. laidlawii* on murine and human lymphocyte cultures

H. KIRCHNER, H. BRUNNER* & H. RÜHL† *Institute of Virology, German Cancer Research Center, Heidelberg, and *Institute of Medical Microbiology, University of Gießen, and † Department of Hematology, Klinikum Steglitz, Free University of Berlin*

(*Received* 10 *January* 1977)

SUMMARY

Heat-inactivated *A. laidlawii* (AL) was found to be a potent mitogen for mouse spleen cells. Spleen cells from homozygous nude mice and spleen cells treated with anti-theta serum and complement responded as well as their respective controls, indicating that AL represented a B-cell mitogen for mouse spleen cells. Spleen cells from LPS-unresponsive C3H/HeJ mice responded well to AL. The peripheral blood leucocytes from unselected human donors were also stimulated by AL, which appeared to represent a T- cell mitogen for human leucocytes. However, the possibility that it acted as a specific antigen could not be excluded. Attention was drawn to the possibility that the presence of mycoplasma might considerably affect the results of tests where tissue-culture cells or derivatives thereof are added to leucocyte cultures.

INTRODUCTION

Mycoplasmas are frequently found as contaminants of tissue-culture cells and represent one of the most serious sources of artifacts in tissue-culture work (MacPherson, 1966). There is an increasing number of tests for cellular immunity in which tissue-culture cells or derivatives thereof are added to leucocyte cultures. In some of these assays such as the mixed-leucocyte tumour-cell interaction (Glaser *et al.*, 1974), antigen recognition is measured by the degree of lymphocyte stimulation induced by inactivated tissue-culture cells.

Previously, it has been shown that certain mycoplasma strains caused inhibition of *in vitro* lymphocyte stimulation (Copperman & Morton, 1966). However, other strains which were not inhibitory were found to be mitogenic (Ginsburg & Nicolet, 1973). We want to demonstrate here stimulation of murine splenic B cells, and of human peripheral T cells by heat-killed preparations of *A. laidlawii*.

MATERIALS AND METHODS

Mycoplasma. A. laidlawii, oral strain, was originally obtained from R.M. Chanock, National Institutes of Health, Bethesda, Maryland, and had been subcultured several times. Its growth medium consisted of 350 ml of PPLO broth (Difco Laboratories Inc., Detroit, Michigan), supplemented with 5 ml of PPLO serum fraction (Difco), 50 ml of 1% yeast extract (Oxoid, Wessel, F.R.G.), 10 ml of 50% glucose, 10 ml of 0·1% phenol red and 12·5 ml of 2% thallium acetate (Chanock, Hayflick & Barile, 1962; Dörner *et al.*, 1976). The pH was adjusted to 7·8 with 1 mol/l sodium hydroxide. *A. laidlawii* was grown at 37°C in screw-capped bottles and harvested by centrifugation for 20 min at 36,000 **g**. The pellet was resuspended in medium RPMI 1640 and heated for 60 min at 56°C. For brevity, heat-killed preparations of *A. laidlawii* will subsequently be designated AL. The amount of protein in the stock preparation used in our experiments was 3·0 mg total protein/ml as determined by the Lowry method (Lowry *et al.*, 1951).

Mouse lymphocyte cultures. C57BL/6J/BOM mice, C57BL/6J/BOM nu–nu mice, and the heterozygous litter mates of the latter were obtained from Gl. Bomholtgard Ltd, 8650 Ry, Denmark. C3H/HeJ and C3HeB/FeJ mice were obtained from Jackson Laboratories, Bar Harbour, Maine.

Single spleen-cell suspensions of these mice were taken up in RPMI 1640, supplemented with foetal bovine serum

Correspondence: Dr Holger Kirchner, Institute of Virology, German Cancer Research Center, 280 Im Neuenheimer Feld, 6900 Heidelberg, F.R.G.

glutamine and antibiotics. All details of the lymphocyte-stimulation assay and the source of the mitogens have been described (Kirchner *et al.*, 1974). Cultures were stimulated with optimal doses of phytohaemagglutinin (PHA), concanavalin A (Con A), *E. coli* lipopolysaccharide (LPS), or with a range of concentrations of AL. [^{3}H]thymidine ([^{3}H]TdR, NET 027, New England Nuclear Co., Boston, Massachusetts, sp. act. 6·7 Ci/mmol) at a final concentration of 1 μCi/ml was added between 45 and 49 hr after initiation of the cultures, which were then collected on filters by using a multiple-harvesting device (Skatron; obtained through Flow Laboratories).

The results were expressed as mean counts per minute per culture of triplicate samples (ct/min).

Treatment of mouse spleen cells by anti-theta serum and complement was performed using the same sera and the same technique as described (Holden, Kirchner & Herberman, 1975).

Human lymphocyte cultures. Heparinized venous blood was collected from unselected, healthy individuals and the mononuclear cells were isolated by Ficoll–Hypaque gradient centrifugation. Approximately 3–5 $\times$ 10^7 cells were passed through nylon-wool columns prepared by the method described by Aisenberg, Lang & Wilkes (1964). The nylon-wool-filtered cells consisted predominantly of T cells forming rosettes with sheep red blood cells (E rosette-forming cells, E-RFC) and contained less than 2% immunoglobulin (Ig)-positive cells or monocytes. To separate E-RFC from non-rosetting cells, neuraminidase-treated sheep red blood cells were mixed with lymphocytes, incubated for 1 hr at room temperature, layered on Ficoll–Hypaque gradients and centrifuged. Cells from the interphase of the gradients consisted predominantly of B cells and contained variable numbers of monocytes; the percentage of E-RFC was less than 2% in all experiments. The pellets contained up to 95% E-RFC and less than 2% Ig-positive cells or monocytes. The pellets were incubated with a red cell lysis buffer to lyse the attached erythrocytes. The percentage of E-RFC was enumerated as described by Pang, Baquley & Wilson (1974); the number of Ig-positive cells was determined by direct immunofluorescence using rabbit anti-human Ig sera (Behringwerke, Germany).

All cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, L-glutamine, and antibiotics. 10^6 cells/ml were cultured in round-bottom microtrays (Greiner AG, Nurtingen, Germany) in a total volume of 0·2 ml/well. PHA (Difco) was added in optimal stimulatory doses, parallel cultures received AL (1:100 dilutions of the stock preparations were found to be optimal). All cultures were prepared in quadruplicate and incubated in an atmosphere of 5% CO_2 in air. PHA cultures were harvested on the third day; optimal stimulation in AL-stimulated cultures was achieved after 6 days. For the last 18 hr the cultures received 1 μCi [^{3}H]TdR/well, harvesting was done as described for mouse lymphocyte cultures. Results are expressed as Δ ct/min (difference between stimulated cultures and untreated control cultures).

RESULTS

Stimulation of mouse spleen cells by AL

AL was found to be markedly mitogenic for spleen cells of C57BL/6 mice. Optimal stimulation was seen at about 18 hr, the dose–response curve was relatively broad with optimal stimulation at a 1:100–1:500 dilution of the stock preparation (data not shown).

Spleen cells of homozygous nude mice were stimulated by AL as well as those of their heterozygous litter mates (Table 1). Stimulation was not abrogated by treatment with anti-theta serum and complement under controlled conditions (Table 2). Spleen cells from C3H/HeJ mice which did not respond to

TABLE 1. Response of spleen cells from heterozygous and homozygous nude mice to AL

	Stimulant*		
Source of spleen cells	None	PHA	AL
Nu/— mouse 1	932	105260	44409
Nu/— mouse 2	332	168772	30641
Nu/— mouse 3	537	153447	22871
Nu/nu mouse 1	4279	3491	31218
Nu/nu mouse 2	2116	2477	39169
Nu/nu mouse 3	843	1348	19714

All results in mean ct/min.

* The concentration of PHA was 1·25 μg/ml, AL was tested at optimally stimulatory doses (30 μg total protein/ml final concentration).

TABLE 2. Effect of treatment by anti-theta serum and complement on the reactivity of C57BL/6 spleen cells to AL

Type of culture	Stimulant*		
	None	PHA	AL
Untreated	432	102419	17939
Treated by complement alone	587	124306	27162
Treated by anti-theta serum plus complement	498	633	28063

All results in mean ct/min.

* PHA and AL, see Table 1.

TABLE 3. Comparison of the mitogenic effect of AL on spleen cells of C3H/HeJ and C3HeB/FeJ mice

Experiment No.	Source of spleen cells	Stimulant		
		None	LPS	AL
1	C3H/HeJ mice	1649	1742	11904
	C3HeB/FeJ mice	2102	40312	25342
2	C3H/HeJ mice	1020	1127	54540
	C3HeB/FeJ mice	1446	81289	57054

All results in mean ct/min.

LPS were reactive to AL while spleen cells from C3HeB/FeJ mice were fully responsive to both LPS and AL (Table 3). Thus, AL appears to be a B-cell mitogen for mouse spleen cells which is not identical with LPS.

Stimulation of human peripheral lymphocytes by AL

AL also represented a good stimulant of human leucocyte cultures. Thus far, we have tested about forty different unselected healthy donors. Leucocyte cultures of all of them responded well to AL (data not shown). Populations of purified T cells containing less than 2% B lymphocytes were obtained by nylon-column passage or by rosette formation and centrifugation through a Ficoll–Hypaque gradient. Purified T cells responded well to AL while populations containing predominantly B cells and less than 2% T cells did not respond (Table 4). Thus, in cultures of human peripheral lymphocytes AL appeared to be a stimulant predominantly of T cells.

TABLE 4. Response of purified populations of human T and B cells to AL

Cell source	PHA	AL
Unfractionated	72615	13630
T-cells*	61753	18092
T-cells†	64839	11187
B-cells†	1617	161

All results are in mean Δ ct/min of five experiments.

* Obtained by nylon-wool column filtration.

† Obtained by E-rosette separation.

DISCUSSION

It has been recently demonstrated that *M. pneumoniae* specifically stimulated human lymphocytes from previously sensitized donors (Fernald, 1972). A report by Biberfeld, Biberfeld & Sterner (1974) has shown that lymphocytes from seronegative control persons also responded to *M. pneumoniae* albeit to a lesser degree than lymphocytes from immune donors. The dose required for stimulation of lymphocytes from non-immune donors was higher than the one which optimally stimulated lymphocytes from seropositive individuals. Although it could not be excluded that a contaminant of the crude mycoplasma preparation was non-specifically mitogenic, the possibility was raised that *M. pneumoniae* acted as a mitogen. A similar observation of a mitogenic effect at high doses of a malaria antigen has been recently reported by Wyler & Oppenheim (1974).

Our own results, using AL, have not been fully conclusive, since there are no data available on the immune status of normal individuals against this species, and since we have had no opportunity to test our blood donors for the presence of specific antibodies. However, the fact that all donors tested thus far have reacted to AL (unpublished data) suggested that AL represented a non-specific mitogen. From the experiments using purified T- and B-cell populations it appeared that AL was a T-cell mitogen for human blood lymphocytes.

Previously, it has been shown that two species of mycoplasma, *M. pulmonis* and *M. pneumoniae*, were mitogenic for spleen cells of rats and mice, respectively (Ginsburg & Nicolet, 1973; Biberfeld & Gronowicz, 1976). *M. pneumoniae* was found to be a B-cell mitogen, or a so-called polyclonal B-cell activator. Our results with AL are in agreement with this report, since spleen cells treated by anti-theta serum plus complement retained full reactivity, and since spleen cells from homozygous nude mice reacted as well as their heterozygous litter mates. In addition, our data strongly suggested that the stimulatory principle of our AL preparation was not LPS since spleen cells of C3H/HeJ mice, which are genetically unresponsive to LPS, responded well to AL. However, it is quite conceivable that the stimulatory principle of AL was a lipoprotein, similar to those which have been recently described to stimulate spleen cells of LPS-unreactive mice (Morrison, Betz & Jacobs, 1976).

The crude preparation of AL which we have been using stimulated both lymphocytes from murine and human origin. LPS, which is the best-characterized mitogen for mouse B cells, practically doesn't stimulate human lymphocytes (Peavy, Adler & Smith, 1970). However, the stimulant from *M. pneumoniae* also appeared to be stimulatory for both human and murine lymphocytes (Biberfeld *et al.*, 1974; Biberfeld & Gronowicz, 1976). Our data have shown that the same preparation of AL which stimulated B cells in murine spleen-cell cultures, predominantly stimulated T cells in cultures of human peripheral blood lymphocytes. It has to be further investigated whether the stimulant for both types of cells is identical or whether there are different mitogens in the crude preparation.

The authors appreciate the dedicated technical assistance of Ms K. Keyssner, Ms D. Erny and Ms G. Bochert. Excellent secretarial assistance was provided by Ms M. Kasamasch.

REFERENCES

AISENBERG, A.C., LANG, J.C. & WILKES, B. (1974) Chronic lymphocytic leukemia cells: rosette formation and adherence to nylon fiber columns. *J. nat. Cancer Inst.* **52**, 13.

BIBERFELD, G., BIBERFELD, P. & STERNER, G. (1974) Cell-mediated immune response following *mycoplasma pneumoniae* infection in man. *Clin. exp. Immunol.* **17**, 29.

BIBERFELD, G. & GRONOWICZ, E. (1976) *Mycoplasma pneumoniae* is a polyclonal B-cell activator. *Nature (Lond.)*, **261**, 238.

CHANOCK, R.M., HAYFLICK, L. & BARILE, M.F. (1962) Growth on artificial medium of an agent associated with typical pneumonia and its identification as a PPLO. *Proc. nat. Acad. Sci. (Wash.)*, **48**, 41.

COPPERMAN, R. & MORTON, H.E. (1966) Reversible inhibition of mitosis in lymphocyte cultures by non-viable mycoplasma. *Proc. Soc. exp. Biol. (N.Y.)*, **123**, 790.

DÖRNER, I., BRUNNER, H., SCHIEFER, H.G. & WELLENSIEK, H.J. (1976) Complement-mediated killing of *Acholeplasma laidlawii* by antibodies to various membrane components. *Infec. Immunity*, **13**, 1663.

FERNALD, G.W. (1972) *In vitro* response of human lymphocytes to *Mycoplasma pneumoniae*. *Infec. Immunity*, **5**, 552.

GINSBURG, H. & NICOLET, J. (1973) Extensive transformation of lymphocytes by a mycoplasma organism. *Nature: New Biology*, **246**, 143.

GLASER, M., HERBERMAN, R.B., KIRCHNER, H. & DJEU,

J.Y. (1974) Study of cellular immune response to Gross virus-induced lymphoma by the mixed lymphocyte-tumor cell interaction. *Cancer Res.* **34**, 2165.

HOLDEN, H.T., KIRCHNER, H. & HERBERMAN, R.B. (1975) Secondary cell-mediated cytotoxic response to syngeneic mouse tumor challenge. *J. Immunol.* **115**, 327.

KIRCHNER, H., HERBERMAN, R.B., GLASER, M. & LAVRIN, D.H. (1974) Suppression of *in vitro* lymphocyte stimulation in mice bearing primary Moloney sarcoma virus-induced tumors. *Cell. Immunol.* **13**, 32.

MACPHERSON, I. (1966) Mycoplasmas in tissue culture. *J. Cell Sci.* **1**, 145.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MORRISON, D.C., BETZ, S.J. & JACOBS, D.M. (1976) Isolation of a lipid bound polypeptide responsible for 'LPS-Initiated' mitogenesis of C3H/HeJ spleen cells. *J. exp. Med.* **144**, 840.

PANG, G.T.M., BAQULEY, D.M. & WILSON, J.D. (1974) Spontaneous rosettes as a T-lymphocyte marker: a modified method giving consistent results. *J. Immunol. Meth*, **4**, 41.

PEAVY, D.L., ADLER, W.H. & SMITH, R.T. (1970) The mitogenic effects of endotoxin and staphyloccocal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. *J. Immunol.* **105**, 1453.

WYLER, D.J. & OPPENHEIM, J.J. (1974) Lymphocyte transformation in human plasmodium falciparum malariae. *J. Immunol.* **113**, 449.