Immunology, 1975, **29**, 697.

Effects of Mitogens for Mouse B Lymphocytes on Chicken Lymphoid Cells

G. TUFVESON AND G. V. ALM

Department of Histology, Institute of Human Anatomy, University of Uppsala, Uppsala, Sweden

(*Received 24th March* 1975; *accepted for publication 9th April* 1975)

Summary. Lipopolysaccharide from *E. coli* (LPS) and purified protein derivative from tuberculin (PPD) increased the [^{3}H]thymidine incorporation of chicken spleen cells in culture. No such stimulation was observed with dextran sulphate. Thymus and bursa lymphocytes were not stimulated by any of these compounds.

Spleen cells from chickens chemically bursectomized with cyclophosphamide treatment showed decreased responses to LPS and PPD, but responded normally to the T-cell mitogen concanavalin A.

None of the tested mitogens induced polyclonal antibody synthesis or directly enhanced the primary antibody response to sheep erythrocytes (SRBC) in spleen cell cultures. LPS-coated SRBC, however, enhanced the *in vitro* antibody response to SRBC.

The results suggest a moderate proliferative response of chicken lymphocytes to LPS and PPD, possibly involving B cells, but no further effects comparable to those on mouse B lymphocytes.

INTRODUCTION

Lipopolysaccharide from *E. coli* (LPS), purified protein derivative from tuberculin (PPD), and dextran sulphate (DxS) are potent mitogens for mouse B lymphocytes (Andersson, Möller and Sjöberg, 1972a; Gery, Kruger and Spiesel, 1972; Sultzer and Nilsson, 1972; Diamantenstein, Vogt, Ruhl and Bochert, 1973; Dörries, Schimpl and Wecker, 1974). These mitogens also induce polyclonal antibody synthesis in mouse spleen cell cultures (Andersson, Sjöberg and Möller, 1972b; Nilsson, Sultzer and Bullock, 1973; Dörries *et al.*, 1974). In addition, they enhance the primary antibody response *in vitro* (Sjöberg, Andersson and Möller, 1972; Kreisler and Möller, 1974; Vogt, Ruhl, Wagner and Diamantenstein, 1973). Furthermore, LPS-coated erythrocytes induce a T cell-independent antibody response to the erythrocytes (Möller, Andersson and Sjöberg, 1972).

Little is known of the effect of these mouse B-cell mitogens on lymphoid cells of avian species. Recent work suggests, however, that LPS induces B-cell mitosis in chicken spleen cell cultures (Weber, 1973). The chicken has a well-defined central lymphoid organ, the bursa of Fabricius, essential for the normal development of B lymphocytes (Gatti, Stutman and Good, 1970; Good, 1972; Cooper, Lawton and Kincade, 1972; Toivanen and

Correspondence: Dr Gunnar Tufveson, Department of Histology, Biomedical Center, Box 571, S-751 23 Uppsala, Sweden.

Toivanen, 1973b). Because the various B-cell mitogens appear to stimulate mouse B cells of different degrees of maturation (Gronowicz, Coutinho and Möller, 1974), they could conceivably also be useful in the analysis of B-cell development in the chicken.

We have therefore investigated *in vitro* effects of LPS, PPD, DxS and the T-cell mitogen (Toivanen and Toivanen, 1973a) concanavalin A (con A) on chicken lymphoid cells. The ability of these mitogens to induce proliferation and polyclonal antibody synthesis as well as enhance the primary antibody response *in vitro* was examined, using mouse lymphocytes as controls. The effect of bursectomy on the mitogen-induced proliferative response of chicken spleen cells was also studied.

MATERIALS AND METHODS

Animals

Five to 8-week-old chickens of the White Leghorn Babcock B-300 strain (Hinseberg Hatchery, Hinseberg, Sweden) and 10-week-old CBA/J mice (Gl. Bomholtgård, Ry, Denmark) were used.

Bursectomy

Chemical bursectomy was performed by intraperitoneal injection of 3 mg of cyclophosphamide (Pharmacia, Uppsala, Sweden) in 0·15 ml of physiological saline (PS) into each chicken daily for 4 days, beginning at the day of hatch (Toivanen, Toivanen and Good, 1972). The efficiency of the cyclophosphamide treatment was checked by determination of the serum immunoglobulin level. For this, the chickens were bled from the wing veins at 4 weeks of age, and serum samples were assayed for immunoglobulin by radial diffusion in agar (Mancini, Carbonara and Heremans, 1965) using a pool of control sera as reference and polyvalent rabbit anti-chicken immunoglobulin as antiserum. Only chickens that had immunoglobulin levels less than 10 per cent of the control level were accepted as bursectomized.

Mitogens

The following mitogens were used: LPS, from *E. coli* 055:85 (Difco, Detroit, Michigan); PPD from tubercle bacteria RT 32 (Statens Seruminstitute, Copenhagen, Denmark); con A (gift from Pharmacia); DxS, obtained as the sodium salt of dextran sulphate 500, molecular weight 5×10^5, with 2·3 sulphate groups per glycosyl residue (Pharmacia). They were all dissolved in PS at a concentration of 1 mg/ml and stored frozen.

Erythrocytes

Sheep erythrocytes (SRBC) were obtained from one single sheep and stored in Acedex with Adenine (Pharmacia) for less than 1 week. Prior to use they were washed five times in PBS (Dulbecco's phosphate-buffered saline) and resuspended in equal parts of PBS and HBSS (Hanks's balanced salt solution) or in culture medium.

Sera

A pool of sera from newly hatched chickens (BCS) was obtained by bleeding non-anesthesized animals from the heart and was heat-inactivated at 56° for 30 minutes. Chicken complement serum was derived from 6–16-week-old surgically bursectomized

chickens. Guinea-pig complement serum was obtained by cardiac puncture. All sera were stored frozen at −80°.

Cell preparations

The chickens were killed by exsanguination from the heart and the mice by cervical dislocation. Their spleens were removed, teased apart with forceps in a plastic Petri dish containing HBSS+PBS. The cell suspensions were transferred into 15-ml plastic test tubes (Falcon Plastics, Oxnard, California) and sedimented for 5 minutes to remove cell aggregates. The supernatants were collected and centrifuged at 400 *g* for 10 minutes.

Thymus cells were either prepared as the spleen cells, or the thymus was cut with scissors and gently passed through a 60-mesh stainless steel screen, while 10 ml of HBSS+PBS were added. Bursa cells were prepared in the latter manner, but in addition the homogenate was passed twice through a 19-G needle mounted on a syringe. The bursa and thymus homogenates were then sedimented and centrifuged as described above for spleen cells, except that bursa cells were washed once in HBSS+PBS. All centrifuged cells were finally resuspended in culture medium, aliquots were stained (Natt and Herrick, 1952), counted in a haemocytometer and the cell concentrations were adjusted.

Chicken cells were prepared at room temperature and mouse spleen cells at +4°.

Culture medium

The cells were cultured in RPMI 1640, containing L-glutamine (2 mM), HEPES-buffer (10 mM) (all from Flow), penicillin (100 u/ml) and streptomycin (100 μg/ml). The culture medium was supplemented with serum only when indicated. Cultures assayed for plaque-forming cells (PFC) were fed daily with 10 μl of a nutritional cocktail described elsewhere (Tufveson, Lundberg and Alm, 1974).

Culture conditions

Cell suspensions in 0·1-ml volumes per culture, containing 1×10^6 chicken cells or $0{\cdot}5 \times 10^6$ mouse cells were cultured in round-bottomed Cooke microtitre plates (Flow) for measurements of DNA synthesis. The cultures were established in triplicates for each treatment. Dilutions of mitogen were added in 0·01-ml volumes per culture to give a final concentration of 0·1, 1, 10 and 100 μg/ml (LPS, PPD and DxS) or 2·5, 5, 10 and 20 μg/ml (con A). Concentrations of 1, 10 and 100 μg/ml (LPS and PPD) and 5 μg/ml (con A) were used in the bursectomy experiment.

Cultures for assays of primary antibody response and polyclonal antibody synthesis were established in triplicate for each treatment as described elsewhere (Tufveson *et al.*, 1974). Briefly, cell suspensions in 0·1-ml volumes per culture were cultured in Falcon Type 3040 Micro-Test II tissue culture plates. The cultures contained $1{\cdot}5 \times 10^6$ mouse spleen cells or $2{\cdot}5 \times 10^6$ chicken cells. 1×10^6 cells per culture were also tried when the chicken spleen cell cultures were assayed for polyclonal antibody synthesis. When indicated the cultures received 2×10^6 SRBC or LPS-coated SRBC (Möller, 1965) in 0·005 ml of culture medium. Mitogens were added as described above.

Chicken cell cultures were incubated at +39·5° and mouse cell cultures at +37° in gastight chambers using a water saturated atmosphere of 95 per cent air and 5 per cent CO_2.

Determination of [^3H]thymidine incorporation

The incorporation of [^{3}H]methylthymidine ([^{3}H]TdR) in the cultures during a 4-hour period on culture day 2 was taken as an indicator of cell proliferation. Preliminary experiments indicated that the mitogen-induced stimulations were maximal at this time. 0·2 μCi of [^{3}H]TdR, specific activity 6·7 Ci/mM (New England Nuclear Corporation, Boston, Massachusetts) in 0·025 ml of PS were added per culture. The pulsed cultures were processed with a Scatron multiple cell culture harvester (Scatron, Lierbyen, Norway). Distilled water was used both to harvest and to wash the cells collected on the glass-fibre filters. The radioactivity was measured by conventional liquid scintillation spectrometry and expressed as counts per minute (ct/minute) per culture.

Assay of antibody-forming cells

The number of direct PFC per culture was determined using the technique of Cunningham and Szenberg (1968) as previously described in detail (Tufveson *et al.*, 1974) but with minor modifications. Briefly, the cells were harvested, washed once, and resuspended in 0·5 ml PBS+HBSS containing 1·2 per cent SRBC or TNP-coupled SRBC (Rittenberg and Pratt, 1969) and either 4 per cent bursectomized chicken serum to develop chicken PFC, or 4 per cent guinea-pig serum to develop mouse PFC. For each assay the cells of three cultures were pooled. 0·1-ml aliquots were incubated in the Cunningham chambers and the plaques were counted using a light microscope.

Statistical treatment

The results of the PFC and radioactivity assays are given as the arithmetic mean (M) plus or minus the standard error of the mean ($\pm$s.e.m.). The results of the bursectomy experiment are presented as the net increase in [^{3}H]TdR incorporation caused by the addition of mitogen (ct/minute/culture). Student's *t*-test was used to evaluate differences between group means.

RESULTS

EFFECT OF MITOGENS ON THE [^{3}H]TdR INCORPORATION OF CHICKEN SPLEEN CELLS

Results of representative experiments designed to test the response of chicken spleen cells at various concentrations of mitogens are shown in Fig. 1.

Both LPS (Fig. 1a) and PPD (Fig. 1b) significantly increased the [^{3}H]TdR incorporation in chicken spleen cell cultures. The responses, which were better in serum-free than in serum-containing medium, were maximal with 10 or 100 μg/ml of LPS and 100 μg/ml of PPD.

DxS, as shown in Fig. 1c did not significantly stimulate the [^{3}H]TdR incorporation at any tested concentration.

Con A (Fig. 1d) stimulated the [^{3}H]TdR incorporation of the cultures both in the absence and presence of 10 per cent BCS. The optimal concentration of con A was usually 5 μg/ml in serum-free cultures and 20 μg/ml in the cultures containing 10 per cent BCS.

EFFECT OF MITOGENS ON THE [^{3}H]TdR INCORPORATION OF THYMUS AND BURSA CELLS

Attempts were made to stimulate thymus and bursa cell cultures with LPS, PPD, DxS and con A, both in the absence (Table 1) and presence of 10 per cent BCS (data not

shown). Only con A, in the absence of serum, stimulated the [^{3}H]TdR incorporation of thymus cell cultures, whereas none of the mitogens stimulated the bursa cell cultures.

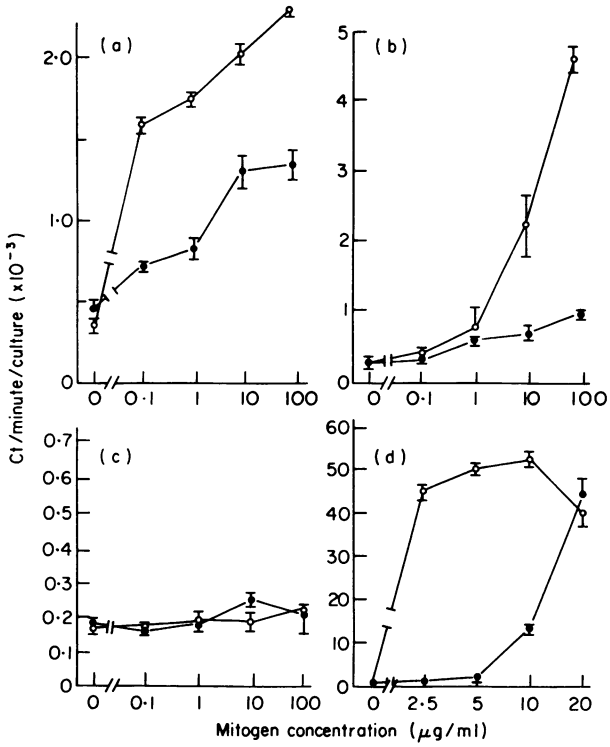

FIG. 1. Incorporation of [^{3}H]TdR (ct/minute/culture; mean ± s.e.m. of triplicate cultures) into chicken spleen cells cultured for 2 days in serum-free (○) and serum-containing (●) (10 per cent BCS) medium with varying concentrations of: (a) LPS; (b) PPD; (c) DxS; (d) con A.

EFFECT OF BURSECTOMY ON THE RESPONSE OF SPLEEN CELLS TO MITOGENS

Spleen cells obtained from six controls and six cyclophosphamide-bursectomized chickens were tested for responsiveness to LPS, PPD and con A in serum-free cultures. The results, expressed as the mitogen-induced net increase in [^{3}H]TdR incorporation, are summarized in Fig. 2. Bursectomy significantly reduced the response to LPS at 1, 10 and 100 μg/ml ($P < 0{\cdot}01, < 0{\cdot}005, < 0{\cdot}005$ respectively) and to PPD at the same concentrations ($P < 0{\cdot}001, < 0{\cdot}025, < 0{\cdot}05$ respectively). The response to con A (5 μg/ml) of spleen cell cultures from the control chickens (44,806 ± 4070 ct/minute/culture) and bursectomized chickens (35,365 ± 4476 ct/minute/culture) did not differ significantly.

EFFECTS OF MITOGENS ON THE [^{3}H]TdR INCORPORATION OF MOUSE SPLEEN CELLS

We considered it important to assess the mitogenic activity of the LPS, PPD and DxS preparations for mouse cells. Table 2 shows that they all stimulated the incorporation of [^{3}H]TdR of serum-free mouse spleen cell cultures. Stimulation was obtained with LPS

and PPD at all concentrations, LPS with a maximum at 10 μg/ml and PPD at 100 μg/ml (the highest concentration tested). DxS stimulated at higher concentrations (10 and 100 μg/ml), but less than LPS and PPD.

TABLE 1

INCORPORATION OF [^{3}H]TdR* INTO CHICKEN THYMUS AND BURSA CELLS CULTURED FOR 2 DAYS IN SERUM-FREE MEDIUM WITH VARYING CONCENTRATIONS OF MITOGENS†

Cell source	Mitogen	Concentration of mitogen (μg/ml)‡				
		0	0·1 (2·5)	1 (5)	10 (10)	100 (20)
Thymus	LPS	157±31*	128±12	219±43	170±24	159±12
	PPD		160±21	242±67	192±5	162±31
	DxS		254±34	245±41	442±173	297±61
	Con A		11,226±1017	12,643±1049	3132±131	1099±43
Bursa of Fabricius	LPS	185±58	82±24	121±43	198±76	118±64
	PPD		224±57	353±30	268±36	179±61
	DxS		223±61	91±21	198±29	140±24
	Con A		153±23	254±68	142±12	253±147

* Ct/minute/culture (mean±s.e.m. of three cultures).
† Cultures supplemented with 10 per cent BCS showed no stimulation.
‡ The figures in parentheses indicate the final concentration of con A only.

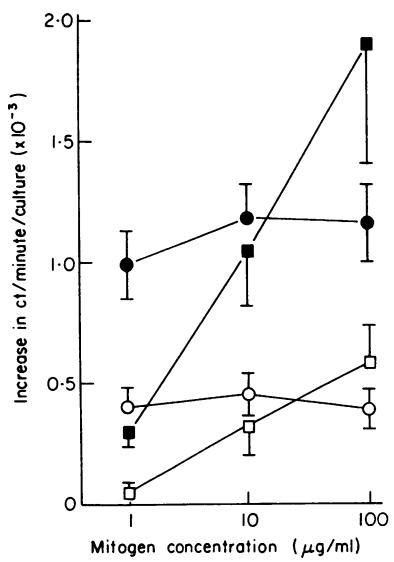

FIG. 2. Net increase in incorporation of [^{3}H]TdR (ct/minute/culture; mean±s.e.m. of six animals) into spleen cells from bursectomized (○, □) and control chickens (●, ■), cultured for 2 days in serum-free medium with varying concentrations of LPS (○, ●) and PPD (□, ■).

ABILITY OF MITOGENS TO INDUCE POLYCLONAL ANTIBODY SYNTHESIS

Attempts were made to induce polyclonal antibody synthesis in chicken spleen cells and bursa cells using LPS, PPD, DxS and con A. Cultures were established both in the absence and presence of 10 per cent BCS. A few control cultures, not receiving mitogen,

were immunized with SRBC. All cultures were assayed for direct PFC on culture day 2 or 3, with SRBC and TNP-coupled SRBC as target cells. The number of TNP–specific PFC was calculated by subtracting the number of SRBC–PFC from the number of TNP–SRBC–PFC.

Serum-containing but not serum-free control spleen cell cultures immunized with SRBC responded with PFC to this antigen on both culture days 2 and 3. None of the mitogens induced any PFC against TNP–SRBC or SRBC in chicken spleen or bursa cell cultures.

TABLE 2

INCORPORATION OF [^{3}H]TdR* INTO MOUSE SPLEEN CELLS CULTURED FOR 2 DAYS IN SERUM-FREE MEDIUM WITH VARYING CONCENTRATIONS OF MITOGENS

Expt no.	Mitogen	Concentration of mitogen (μg/ml)				
		0	0·1	1	10	100
1	LPS	283 ± 12*	2964 ± 88	10,162 ± 230	25,283 ± 833	15,510 ± 639
	PPD		356 ± 9	1678 ± 55	5613 ± 38	8232 ± 208
2	DxS	448 ± 23	549 ± 71	549 ± 1	1048 ± 71	2505 ± 224

* Ct/minute/culture (mean ± s.e.m. of three cultures).

TABLE 3

NUMBER OF TNP-SPECIFIC PFC* IN MOUSE SPLEEN CELLS CULTURED FOR 4 DAYS IN SERUM-FREE MEDIUM WITH VARYING CONCENTRATIONS OF MITOGENS

Mitogen	Concentration of mitogen (μg/ml)				
	0	0·1	1	10	100
LPS	10*	35	117	230	214
PPD		3	3	75	177
DxS		8	5	10	12

* PFC per culture (three cultures were pooled for the assay).

As a control, serum-free mouse spleen cell cultures were stimulated with LPS, PPD and DxS. Table 3 shows that both LPS and PPD induced TNP-specific PFC, while DxS only gave a small number of PFC.

EFFECT OF MITOGENS ON THE PRIMARY ANTIBODY RESPONSE OF CHICKEN SPLEEN CELLS

LPS, PPD, DxS and con A were added to chicken spleen cell cultures immunized with SRBC. The cultures were assayed on culture day 3 for direct PFC with SRBC as target cells (Table 4).

None of the mitogens improved the PFC response. Con A (10 and 20 μg/ml) and DxS (100 μg/ml) completely inhibited the PFC response, whereas LPS (100 μg/ml) and PPD (100 μg/ml) clearly reduced the response. PFC were not induced in the absence of 10 per cent BCS (data not shown).

TABLE 4

PRIMARY ANTIBODY RESPONSE TO SRBC* IN CHICKEN SPLEEN CELLS CULTURED FOR 3 DAYS IN MEDIUM WITH 10 PER CENT BCS AND VARYING CONCENTRATIONS OF MITOGEN. ALL CULTURES CONTAINED 2×10^6 SRBC†

Mitogen	Concentration of mitogen (μg/ml)‡				
	0	0·1 (2·5)	1 (5)	10 (10)	100 (20)
LPS	749 ± 167§	1005¶	1175	605	330
PPD		510	715	1110	95
DxS		960	950	125	0
Con A		585	175	0	0

* Measured as PFC per culture with SRBC as target cells in the PFC assay.
† PFC were not detected in the absence of serum (10 per cent BCS) or SRBC.
‡ The figures in parenthesis indicate the final concentration of con A.
§ PFC per culture (mean ± s.e.m. of four triplicate cultures).
¶ PFC per culture (three cultures were pooled for the assay).

TABLE 5

PRIMARY ANTIBODY RESPONSE* BY CHICKEN SPLEEN CELLS CULTURED FOR 3 DAYS IN MEDIUM WITH 10 PER CENT BCS AND EITHER 2×10^6 SRBC OR LPS-COATED SRBC PER CULTURE

Animal number	Antigen in the cultures	
	SRBC	LPS-coated SRBC
1	51 ± 11†	455 ± 61
2	134 ± 51	255 ± 28
3	498 ± 67	920 ± 90
4	26 ± 3	126 ± 11

* Measured as PFC per culture with SRBC as target cells in the PFC assay.
† PFC per culture (mean ± s.e.m. of three triplicate cultures).

LPS-COATED SRBC AS ANTIGEN IN THE PRIMARY ANTIBODY RESPONSE

Chicken spleen cell cultures were immunized with either SRBC or LPS-coated SRBC and assayed for direct PFC with SRBC as target cells on culture day 3. The PFC responses were considerably better with LPS-coated SRBC than with SRBC (Table 5).

DISCUSSION

The present investigation demonstrates that the mouse B-cell mitogens LPS and PPD, but not DxS, significantly stimulate cell proliferation in chicken spleen cell cultures. The responses obtained were even under optimal serum-free culture conditions modest compared to the stimulation with con A, a T-cell mitogen in the chicken (Toivanen and Toivanen, 1973a). Furthermore, LPS and PPD stimulated mouse spleen cells considerably better than chicken spleen cells, although the dose–response curves in serum-free cultures were similar.

LPS and PPD are generally considered to be mitogenic for mouse B lymphocytes (Andersson *et al.*, 1972a; Gery *et al.*, 1972; Sultzer and Nilsson, 1972). We obtained some evidence that they may also stimulate chicken B cells. Thus, thymus lymphocytes, predominantly T cells, were stimulated by the T-cell mitogen con A, but not by LPS and PPD. In addition, cyclophosphamide-induced bursectomy significantly decreased the proliferative response of spleen cells to LPS and PPD (by 65 and 70 per cent respectively). The inhibitory effect of bursectomy was therefore not complete. It is likely that the observed residual response was due to remaining B cells, because the bursectomized birds were not completely agammaglobulinaemic. It could also represent a weak stimulation of peripheral T lymphocytes.

The possibility that cyclophosphamide treatment also damaged the T-cell system (Seto, 1970; Eskola and Toivanen, 1974) was less likely because the bursectomized birds used in our study showed normal *in vitro* responses to the T-cell mitogen con A.

Bursa cells, an almost pure population of B cells (Hudson and Roitt, 1973), did not respond to either LPS, PPD or con A. The reason for this can be the poor survival of these cells *in vitro* (Weber and Eichholtz, 1971; our unpublished observations). Bursa cells may also represent a differentiation stage with poor mitogen reactivity. Relevant to this is the suggestion (Gronowicz *et al.*, 1974) that the B-cell mitogens stimulate mouse B cells of different maturity.

LPS, PPD and DxS did not directly enhance the *in vitro* primary antibody response to SRBC. This is in contrast to the effect of these mitogens on mouse spleen cell cultures (Sjöberg *et al.*, 1972; Armerding and Katz, 1974; Kreisler and Möller, 1974; Vogt *et al.*, 1973). We noted that LPS and PPD at high concentrations (100 μg/ml) and DxS also at lower concentration (10–100 μg/ml) actually suppressed the PFC response. Whether this suppression is related or not to the very efficient con A-induced inhibition of the primary antibody response of the chicken spleen cells found in this study and of mouse spleen cells (Dutton, 1972; Rich and Pierce, 1973) was not determined.

Our previous finding that the *in vitro* primary antibody response by chicken spleen cells was stimulated by allogeneic spleen cells (Tufveson *et al.*, 1974) and the present observation that LPS-coated SRBC improved the *in vitro* antibody response suggest that at least some non-specific enhancement of B-lymphocyte function in the chicken is possible. The latter finding may be interpreted in terms of a conversion of SRBC into a thymus-independent antigen (Möller *et al.*, 1972) but other explanations, such as improved T-cell or macrophage function (Armerding and Katz, 1974) are conceivable.

Finally, we were unable to detect any induction of polyclonal antibody synthesis by LPS, PPD or DxS in chicken spleen or bursa cell cultures. This is in marked contrast to the effect of these mitogens on mouse spleen lymphocytes (Andersson *et al.*, 1972b; Nilsson *et al.*, 1973; Dörries *et al.*, 1974).

The limited effect of the employed B-cell mitogens on chicken lymphocytes in the present study cannot be attributed to a generally poor stimulatory activity in our hands, because in the control experiments they were potent stimulators of mouse spleen cells. Furthermore, the culture conditions for chicken cells appear adequate and well support responses to T-cell mitogens as well as the primary antibody response to SRBC.

On the basis of the results of the present investigation and a previous study (Weber, 1973) it therefore appears reasonable that LPS and PPD are weak mitogens for chicken B lymphocytes, but that some effect on T cells cannot be excluded. The weak proliferative response as well as the lack of further effects, such as polyclonal activation of antibody

synthesis and enhancement of the primary antibody response may either be an inherent property of chicken B cells, e.g. lack of appropriate mitogen receptors, or a property imposed on B cells, e.g. by suppressor T cells. The latter suggestion would be even more critical if LPS, PPD or DxS also to some extent activate chicken T cells and motivates further studies of the effect of these mitogens on T cell-depleted spleen cells.

ACKNOWLEDGMENTS

The competent technical assistance of Mrs Eva Beckman and Mrs Siv Johansson is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (number B75-16X-33-12), Socialstyrelsens Prenatalforskningsnämnd and the Medical Faculty of the University of Uppsala.

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