Immunology 1976 **30** 367

Enumeration of polyclonal mitogen-responsive cells in different lymphoid tissues of the mouse

M. J. DOENHOFF,* G. JANOSSY† & R. S. KERBEL‡ *Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London*

Received 18 *August* 1975; *accepted for publication* 3 *September* 1975

Summary. The relative number of cells capable of responding to Con A, PHA and LPS in the spleen, blood, lymph node and Peyer's patches of CBA mice has been quantified by means of a cytological analysis technique. No difference has been found between Con A- and PHA-responsive cells in spleen and lymph node. The lymphoid tissues of T cell-deprived mice have a reduced content of PHA responsive cells, but LPS responsiveness is within normal limits. Pretreatment of peripheral lymphocyte populations with high concentrations of anti-θ antiserum and complement abolishes the response of the treated cells to PHA, but not to LPS, whereas similar treatment with a cytotoxic anti-immunoglobulin serum, which has no effect on PHA-responsive cells, only partially reduces the response to LPS. The results for mitogen responsiveness are discussed with reference to other methods of quantifying T and B cells using cell-surface markers.

INTRODUCTION

The feasibility of quantifying blood-borne PHA-responsive cells by means of a chromosome marker technique has been demonstrated previously (Doenhoff, 1971), and this method has been subsequently used to study the time-dependent regeneration of the T-cell pool in thymectomized, irradiated, bone marrow-reconstituted mice given a thymus graft (Doenhoff and Davies, 1971).

Two limitations of the method at that stage of development were that it gave little information about the state of an animal's lymphoid system apart from the blood—it is apparent that small variations in lymphocyte populations of the spleen and lymph node could disproportionately affect the size of the blood lymphocyte pool; and also that the method was only applicable to quantification of mitogen-responsive T cells and gave no information about the B-cell pool. There was the advantage, however, that blood samples could be taken sequentially without killing the donor animal (Doenhoff, Davies, Leuchars and Wallis, 1970).

PHA and concanavalin (Con A) have been shown to be relatively specific in stimulating mouse T cells to incorporate radioactive thymidine (Greaves and Janossy, 1972; Stobo, Rosenthal and Paul, 1972) and to divide (Doenhoff *et al.*, 1970; Doenhoff, Janossy, Greaves, Gomer and Snajdr, 1974) in both spleen and blood, whereas bacterial lipopolysaccharide (LPS) is a specific mitogen for spleen B cells (Gery, Krüger and Spiesel, 1972; Doenhoff *et al.*, 1974) albeit possibly acting only on a subpopulation of this lymphocyte type (Greaves, Janossy, Feldmann and Doenhoff, 1974a).

The purpose of this paper is to explore further the possibilities of the quantification system based

* Present address and correspondence: Immunology Laboratory, Department of Medical Helminthology, London School of Hygiene and Tropical Medicine, Winches Farm Field Station, 395 Hatfield Road, St Albans, Herts.

† Present address: Department of Pathology, University College Hospital Medical School, University Street, London WC1.

‡ Present address: Department of Pathology, Queen's University, Kingston, Ontario, Canada K7L 3N6.

on chromosome marker analysis, using spleen cells as a standard for comparing the responsiveness of other CBA mouse lymphoid tissues to both T- and B-cell specific mitogens. In addition the capacity of cells to respond to such mitogens has been studied after they had been treated with specific cytotoxic antisera and complement. The quantitative results for the mitogen responsiveness of T and B cells, and their relative susceptibilities to cytotoxic antisera are compared with each other.

MATERIALS AND METHODS

Mice

Eight to 20-week-old mice of the CBA/Lac (hereafter called Lac) and CBA/H.*T6T6* (T6T6) strains of both sexes were used throughout.

The methods of adult thymectomy, administration of 850 rad total body irradiation, and reconstitution with 5×10^6 femoral bone marrow cells are given elsewhere (Davies, Leuchars, Wallis and Koller, 1966).

Preparation of cell suspensions

Cells from spleen, lymph node and Peyer's patches were teased out from the constraining capsular material by squeezing the tissue (about 10 mg of spleen, all the lymph node or three to four Peyer's patches) between a pair of scissors and the inside of a 2·5-ml culture tube in approximately 0·05 ml of culture medium. This method of teasing out cells resulted in a cell viability above 90 per cent as adjudged by trypan blue exclusion. The cell suspension so obtained was diluted by addition of 0·5–1·0 ml of medium, and the larger tissue fragments were allowed to settle for a few minutes before the overlying suspension was transferred to a fresh culture tube. The latter tube was centrifuged gently and the cells resuspended in fresh medium prior to a cell count being made and transfer of an appropriate number of cells to the final culture vessel.

Peripheral blood lymphocytes were obtained by defibrination and plasma gel (Roger Bellon, Neuilly, France) induced erythrocyte sedimentation as described previously (Festenstein, 1968; Doenhoff *et al.*, 1970). Thoracic duct lymphocytes were obtained from anaesthetized mice, force-fed with 0·5 ml of vegetable oil 2 h previously. The thoracic duct was exposed with the aid of retractors via a transverse incision in the left flank of the animal, and ruptured to allow lymph to pass into the abdominal cavity from where it was aspirated. Lymph was collected over a period of 1 h.

Counts of nucleated cell suspensions were made by means of a Coulter Counter Model B with Zaponin to lyse contaminating erythrocytes.

Lymphocyte culture

For culture, the final cell density was in all cases 2×10^6 cells per millilitre of medium. The culture medium was RPMI 1640 with Na_2HCO_3 buffer (Flow) and 10 per cent heat-inactivated foetal calf serum, the latter being obtained either from Flow, or from Difco Laboratories in lyophilized form. Antibiotics in the form of penicillin derivatives were added as well as 300 mM glutamine.

Cultures for cytological analysis were incubated for 70 h at 37° in an atmosphere of 4 per cent CO_2: 10 per cent O_2 and nitrogen. These cultures contained 1×10^6 cells in 0·5 ml of medium held in flat based culture tubes. Cultures for the measurement of radioactive thymidine uptake were prepared in microplate tiles as described by Janossy, Greaves, Doenhoff and Snajdr (1973).

Mitogens used were: con A obtained from Miles Yeda, Israel, or from Pharmacia, added to the culture medium at a concentration of 3 μg/ml; phytohaemagglutinin (Burroughs Wellcome purified grade), 1·5 μg/ml; bacterial lipopolysaccharide (lipopolysaccharide B, *E. coli* O55:B5; Difco), 50–100 μg/ml. For cytological analysis, Colcemid (Ciba) was added at a concentration of 10^{-7} M some 12–16 h before harvesting, and metaphase spreads of the dividing cells obtained according to the method described by Ford (1966).

Radioactive thymidine (Radiochemical Centre, Amersham) of low specific activity (50 mCi/mmol) was used for thymidine utilization assays and added to the microplate cultures at a concentration of 4 μCi/ml. The cultures were harvested as described by Janossy *et al.* (1973).

Antisera and cytotoxic tests

Anti-θ_{C3H} antiserum was prepared in AKR mice and used with fresh guinea-pig serum as a complement source in a cytotoxic assay as described by Kerbel, Elliott and Wallis (1974). Rabbit anti-mouse immunoglobulin serum was produced and used as described by Kerbel and Doenhoff (1974). For experiments involving anti-θ and anti-immunoglobulin sera, aliquots of spleen or lymph node cells

(10–15 × 10⁶ cells) were treated with the respective antiserum and fresh guinea-pig complement. The cells were washed once, recounted, and the appropriate number mixed with an equal number of chromosomally distinct spleen cells for culture with mitogen as above. The percentage of cells killed by the antiserum and complement was determined in a trypan blue dye exclusion test using the formula: [(Percentage dead cells in cytotoxic serum – percentage dead cells in normal serum [(100 – percentage dead cells in normal serum)] × 100.

RESULTS

Normal mice

Suspensions of spleen cells from untreated Lac and T6T6 mice were prepared at final concentrations of 2×10^6/ml of culture medium. The two suspensions were mixed so that cultures contained either equal numbers of each cell type, or 25 per cent of one type and 75 per cent of the other and they were stimulated with either Con A or LPS. The cultures were harvested for cytological analysis after 3 days incubation and the results are given in Fig. 1.

The percentage of dividing cells of each karyotype in Con A stimulated cultures (Fig. 1a) correlated well with the 45° line passing through the origin, this line representing the value to be expected if spleens from the two strains of mice contain the same proportion of Con A-responsive cells. In the LPS response (Fig. 2b), Lac mice had a slightly higher proportion of responding cells in the spleen than T6T6 mice. Nevertheless, as with Con A, the observed values for dividing cells of each type correlated reasonably well with the relative proportions of cells of each type originally put into culture.

Spleen cells from T6T6 mice were then used as a standard to ascertain the relative proportions of Con A-, PHA- and LPS-responsive cells in the blood-borne lymphocyte pool, and in mesenteric lymph nodes and Peyer's patches of Lac mice. Thus the respective nucleated cell suspensions were prepared and again mixed in 1:3, 1:1, and 3:1 ratios with spleen cells and the results of cytological analysis after mitogenic stimulation of the mixtures are given in Fig. 2a, b and c for the three tissues.

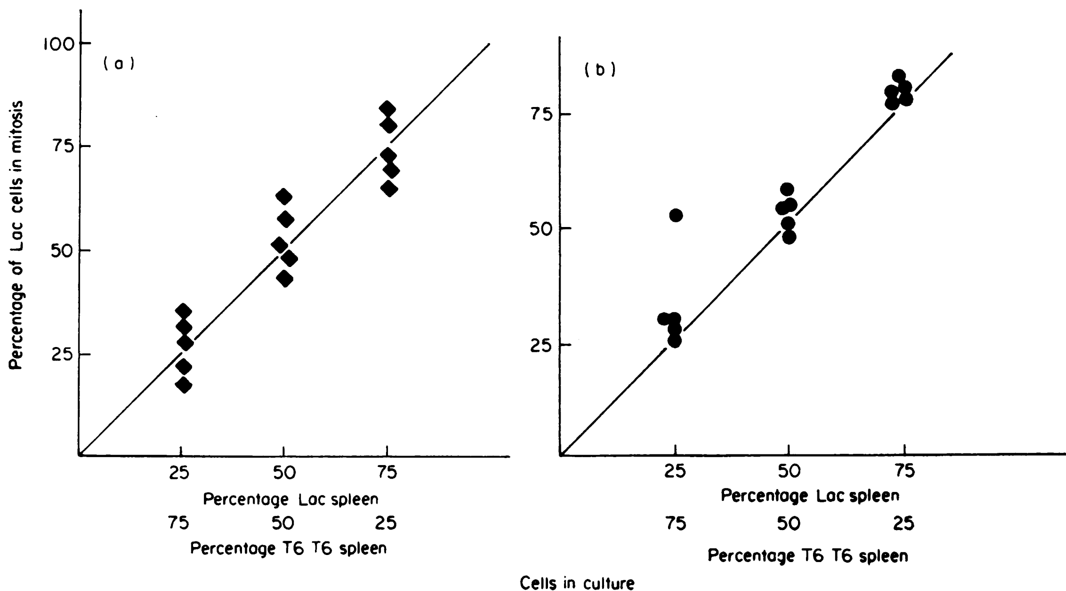

Figure 1. (a) The percentage of Lac karyotype metaphases observed in Con A-stimulated cultures containing Lac and T6T6 nucleated spleen cells mixed before culture in ratios of 1:3, 1:1 and 3:1 respectively. (Each symbol in Figs 1 and 2 represents the value obtained from one, or the mean of two cultures set up with cell populations isolated on separate occasions.) (b) The percentage of Lac karyotype metaphases observed in LPS-stimulated cultures containing Lac and T6T6 nucleated spleen cells mixed before culture in ratios of 1:3, 1:1 and 3:1 respectively.

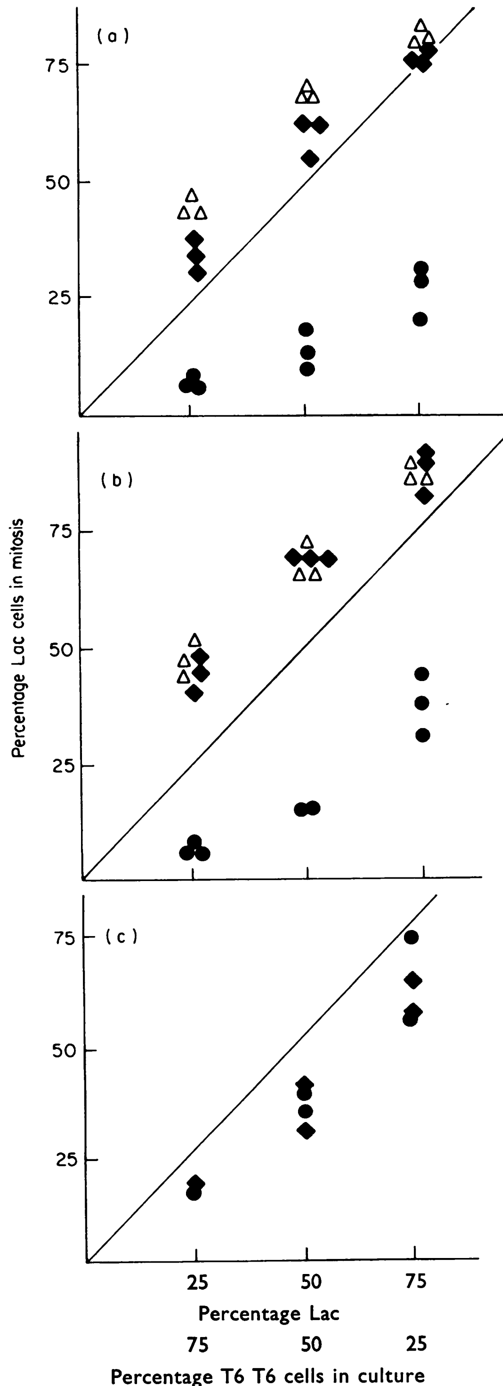

Figure 2. (a) The percentage of Lac karyotype metaphases observed in PHA (△), Con A (⧫) and LPS (●) stimulated cultures, containing T6T6 nucleated spleen cells and Lac nucleated peripheral blood cells mixed before culture in ratios of 1:3, 1:1 and 3:1 respectively. (b) The percentage of Lac karyotype metaphases in PHA (△), Con A (⧫) and LPS (●) stimulated cultures, containing T6T6 nucleated spleen cells and Lac nucleated mesenteric lymph node cells mixed before culture in ratios of 1:3, 1:1 and 3:1 respectively. (c) The percentage of Lac karyotype metaphases observed in Con A (⧫) and LPS (●) stimulated cultures containing T6T6 nucleated spleen cells and Lac nucleated Peyer's patch cells mixed before culture in ratios of 1:3, 1:1 and 3:1 respectively.

Both blood and mesenteric lymph nodes contain proportionately greater numbers of cells responsive to T-cell mitogens than spleen, and fewer LPS-responsive cells. Peyer's patches relative to spleen are deficient in both Con A- and LPS-responsive cells. In cultures containing mesenteric lymph node cells there is no difference between the responses to PHA and Con A, this being unexpected in view of the claim by Stobo and Paul (1973) that mouse spleen but not lymph node contains a Con A-responsive cell population which is not responsive to PHA.

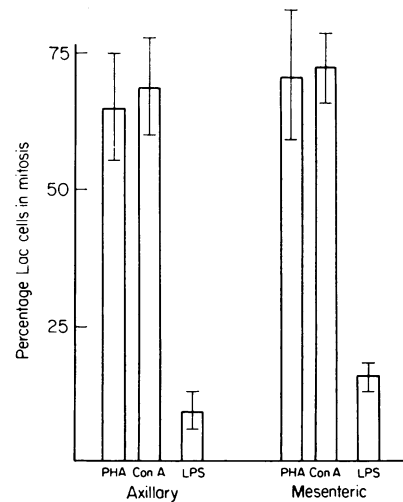

Figure 3. The percentage of Lac cells observed in metaphase in PHA-, Con A- and LPS-stimulated cultures of axillary or mesenteric lymph node cells obtained from untreated Lac mice and mixed with an equal number of normal T6T6 spleen cells before culture. Each histogram bar is the mean value ± the standard deviation of the results for the lymph nodes of four Lac mice.

Fig. 3 indicates that there is no difference between mesenteric and axillary lymph node cells in their response to T-cell mitogens, although mesenteric

nodes contain a marginally greater number of LPS-responsive cells than axillary nodes.

Equivalent cell mixtures to those analysed cytologically (Fig. 2) were assayed for radioactive thymidine uptake. With respect to Con A-stimulated cultures (Fig. 4a), only Peyer's patch cells were somewhat less responsive to this mitogen than spleen cells when they were cultured by themselves, as might perhaps be expected from the cytological analysis result in Fig. 2c. However, blood and lymph nodes cultured alone were approximately as responsive as spleen in terms of their ability to utilize thymidine when stimulated by Con A.

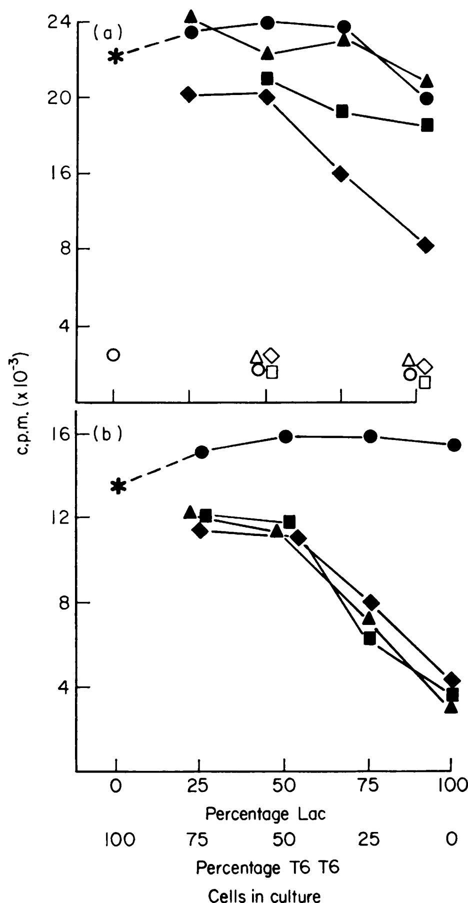

Figure 4. (a) The degree of incorporation of radioactive thymidine (in counts per minute per 0·25-ml volume) by Con A-stimulated cultures containing normal T6T6 nucleated spleen cells mixed with nucleated Lac spleen (●), mesenteric lymph node (▲), blood (■) and Peyer's patch (◆) cells. * = Control for cultures with T6T6 spleen cells alone. Open symbols are the values for thymidine incorporation observed in equivalent cultures without mitogen. These results were obtained using the same populations of cells as were used in Fig. 2. (b) The degree of incorporation of radioactive thymidine (in counts per minute per 0·25-ml volume) by LPS-stimulated cultures of normal T6T6 and Lac lymphoid cells. Symbols are the same as in (a). These results were obtained from the same cell isolates as were used for the results in Fig. 2.

The response to LPS measured by thymidine uptake (Fig. 4b) was substantially reduced in cultures containing lymphocytes derived from blood, lymph node, and Peyer's patches, particularly when the spleen cells contributed less than 50 per cent to the cultured mixture. This is to be expected from the cytological analysis results (Fig. 2) which showed that blood, lymph node and Peyer's patches contained relatively few LPS-responsive cells compared to spleen.

T cell-deprived and reconstituted mice

It is apparent from the preceding experiments that results subject to reasonable interpretation can be obtained using a spleen cell population as a standard for determining the relative numbers of mitogen-responsive cells in other lymphoid tissues. To test the system further, lymphoid tissues from three CBA mouse groups were taken for culture. In one group the mice were untreated (normal controls), whereas the other two had been given 850 rad total body irradiation and 5×10^6 Lac bone marrow cells, with their thymus either intact (T cell-reconstituted group), or removed (T cell-deprived group). Cell suspensions from the peripheral lymphoid tissues of these mice were prepared for culture some 70 days after irradiation, and these cells were mixed with an equal number of spleen cells from an untreated T6T6 mouse. These mixtures were stimulated with either PHA or LPS and the results for cytological analysis of the dividing cells harvested after 3 days of culture are given in Fig. 5a (PHA) and 5b (LPS).

It can be seen that the cells from the four lymphoid tissues of untreated Lac mice gave a result which compares favourably with those observed in similar 50/50 mixtures in Figs 1 and 2. As might be expected adult thymectomy followed by irradiation and bone marrow reconstitution (T cell-deprived mice) has diminished the PHA-responsive cell pool by 70–80 per cent in all four tissues, though it is to be noted that the LPS-responsive cells from these mice did not show much of a compensatory increase in their

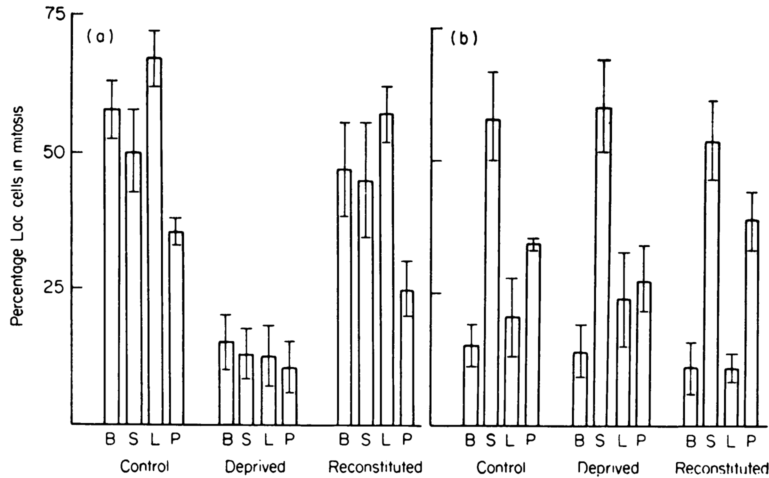

Figure 5. (a) The percentage of Lac cells observed in metaphase in PHA-stimulated cultures containing nucleated cells from blood (B), spleen (S), mesenteric lymph node (L) and Peyer's patches (P) of untreated (control), T cell-deprived and T cell-reconstituted Lac mice. Lymphoid cells from five mice in each group were mixed separately with an equal number of normal T6T6 spleen cells before culture. The results given are the mean value ± the standard deviation of the results for each tissue. (b) The percentage of Lac cells found in metaphase in LPS-stimulated cultures of the same cells as in Fig. 5a.

numbers (see results in Table 1). Irradiated mice with an intact thymus had somewhat smaller PHA-responsive cell pools than untreated controls, in agreement with previous results (Doenhoff, 1971) which indicated that even 70 days after irradiation the T-cell pool was still not fully reconstituted.

Comparison of germ-free and conventional mice

Thymus-dependent areas and θ-positive cells have been identified in Peyer's patches (Parrott and Ferguson, 1974; Waksman, 1973; Raff, 1971), but in contrast to blood and lymph nodes, Peyer's patches contained fewer mitogen-responsive cells (of both types) relative to spleen (Figs 2c and 5). This prompted the question whether this was due to some Peyer's patch cells being involved in gut-associated immune reactions, thus perhaps rendering them unresponsive to *in vitro* mitogen stimulation. Although such immune reactions are probably by no means absent in germ-free mice given solid food, it was considered that the absence of bacteria in the gut of these animals may affect the proportions of mitogen-responsive cells in the Peyer's patches. To this end, mesenteric lymph node and Peyer's patch cells from adult germ-free and conventional T6T6 mice were independently mixed with an equal number of spleen cells from a conventional Lac mouse and stimulated in culture with PHA or LPS.

The results for cytological analysis are given in Fig. 6. It can be seen that both tissues from germ-free mice contained proportionately fewer PHA-responsive cells than their conventional counterparts. There was little difference in the content of LPS-

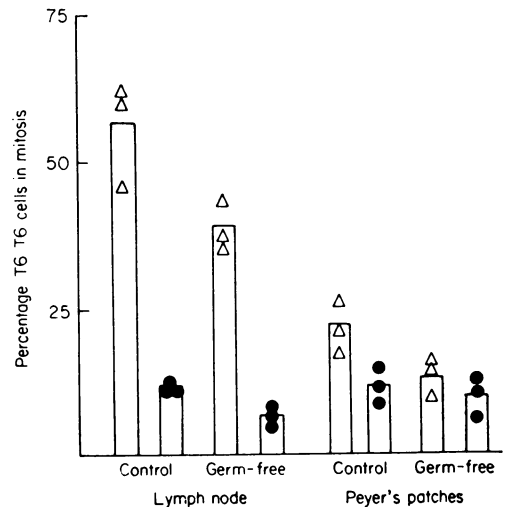

Figure 6. The percentage of T6T6 cells observed in metaphase in PHA (Δ) and LPS (●) cultures of mesenteric lymph node and Peyer's patch cells from conventional (control) or germ-free T6T6 mice, mixed with an equal number of normal Lac spleen cells before culture. Each histogram bar represents the mean value obtained from tissues of three mice.

responsive cells of Peyer's patches of the two types of T6T6 mice, but the relative number of this cell type in the mesenteric nodes of the germ-free animals was at a level which is comparable to that found in axillary nodes of conventional animals (Fig. 3). This observation may be attributable to a relative absence of immunological activity in germ-free mesenteric and conventional axillary lymph nodes. The deficiency in T-cell mitogen-responsive cells in both lymph nodes and Peyer's patches of germ-free mice may also be due to a generally underdeveloped lymphoid system as a result of reduced stimulation of these animals with antigenic matter.

The effects of anti-θ and anti-immunoglobulin antisera

As well as PHA and LPS responsiveness being potentially useful for identification and quantification of T and B cells, albeit subpopulations of both, tests based on cell membrane antigenic differences can also be effectively used to discriminate between these two cell types. Thus the θ isoantigen has been used to quantify T cells in peripheral lymphoid tissues (Raff, 1971) and more recently, rabbit anti-mouse immunoglobulin serum has been shown to be cytotoxic for mitogen-responsive B lymphocytes (Forman and Möller, 1973; Gorczynski and Rittenberg, 1974) as well as having been in use for some time for identifying B cells in ultraviolet (Raff, Sternberg and Taylor, 1970) and in electron microscopy (de Petris and Raff, 1972). In an attempt to relate mitogen responsiveness of cell populations with their surface membrane antigen characteristics, lymphocyte suspensions were pretreated with specific antisera and complement, and both the percentage of cells killed in a trypan blue dye exclusion test and the residual mitogen responsiveness were determined.

Thus Lac spleen and lymph node cells were treated with four-fold dilutions of anti-θ antiserum and guinea-pig complement and stimulated with PHA or LPS in the presence of equal numbers of untreated T6T6 spleen cells. The results for the percentage of cells killed by the serum, and for mitogen responsiveness of the treated Lac cells are given in Fig. 7a (spleen) and b (lymph node). It can be seen that whereas the proportion of LPS-responsive cells remained unaffected by prior treatment with anti-θ serum, nearly all of the PHA

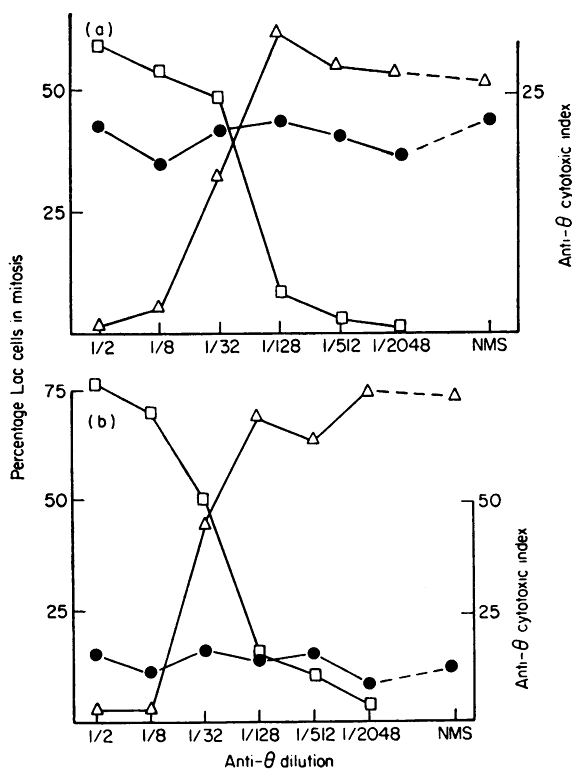

Figure 7. (a) The percentage of Lac cells observed in metaphase in PHA (△) and LPS (●) stimulated cultures following treatment of Lac spleen cells with four-fold dilutions of anti-θ_{C3H} serum, or a 1:1 dilution of normal mouse serum (NMS), followed by guinea-pig complement, and mixing the treated cells with an equal number of normal T6T6 spleen cells before culture. (□) Cytotoxic Index for each dilution of anti-θ serum on the Lac spleen cells used for culture, assessed by means of the trypan blue dye exclusion test. (b) As in (a), but substituting normal Lac mesenteric lymph node cells for Lac spleen cells.

responsiveness of both spleen and lymph node was abrogated by serum used at dilutions of up to 1 in 8.

The results observed when Lac spleen cells were treated with anti-immunoglobulin serum and complement are given in Fig. 8. Even the highest concentration of this serum was unable to remove all LPS-responsive cells, in spite of the fact that the serum killed as many cells as are normally stained with fluorescent anti-immunoglobulin serum in ultraviolet microscopy (Greaves, Owen and Raff, 1973). Forman and Möller (1973) also have reported that they were unable to reduce the LPS response of cells to control unstimulated levels by treatment with anti-immunoglobulin serum. The fact that a

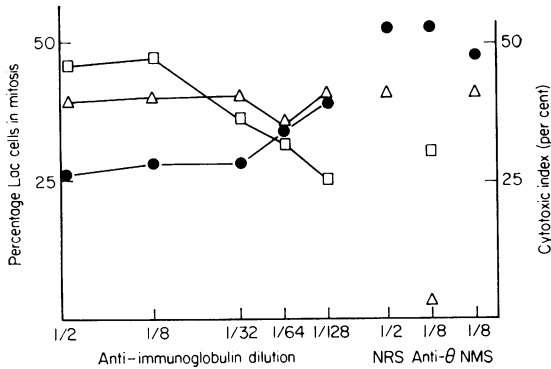

Figure. 8. The percentage of Lac cells observed in metaphase in PHA (Δ) and LPS (●) stimulated cultures following treatment of Lac spleen cells with dilutions of rabbit anti-mouse immunoglobulin antiserum or normal rabbit serum (NRS) or anti-θ serum or normal mouse serum (NMS), and guinea-pig complement. The serum-treated cells were mixed with an equal number of normal T6T6 cells prior to culture. (□) Cytotoxic Index of the Lac spleen cells at each antiserum dilution, assessed by means of the trypan blue dye exclusion test.

1 in 32 dilution of anti-immunoglobulin serum, although being less cytotoxic than 1 in 8 or 1 in 2 dilutions, still caused the maximum observed suppression of LPS responsiveness might suggest that the serum was acting against more than one spleen cell subpopulation. With respect to the controls, anti-θ serum diluted 1 in 8 caused almost total suppression of PHA responsiveness and was cytotoxic for 30·5 per cent of these spleen cells, but did not affect the response to LPS.

It might have been supposed that because both the anti-θ and anti-immunoglobulin sera reduced the number of viable cells in the suspensions used subsequently for culture, the proportion of LPS and PHA-responsive cells respectively should have increased relative to untreated cells used as the standard. However, since the cells were counted electronically, and their numbers were not found to be substantially reduced following antiserum treatment, this method of counting may not distinguish between dead and living cells. It was therefore supposed that if a lymph node cell population that had been treated with anti-θ serum and complement

Table 1. Spleen and mesenteric lymph node (LN) cells from T6T6 and Lac mice were treated with a 1 in 8 dilution of anti-θ antiserum and guinea-pig complement, or left untreated, and a subsequent estimate of viable cell number made by trypan blue exclusion

Proportion of viable cells in cultured mixture (per cent)							Proportion of cells in metaphase after 3 days culture	
T6T6			Lac			Mitogen	T6T6 (per cent)	Lac (per cent)
Tissue	Anti-θ	Per cent	Tissue	Anti-θ	Per cent			
Spleen	−	50	Spleen	−	50	LPS	48	52
Spleen	+	50	Spleen	+	50	LPS	53	47
Spleen*	−	75	LN	−	25	LPS	92	8
Spleen*	−	50	LN	−	50	LPS	73	27
Spleen*	−	25	LN	−	75	LPS	56	44
Spleen	−	75	LN	+	25	LPS	92	8
Spleen	−	50	LN	+	50	LPS	72	28
Spleen	−	25	LN	+	75	LPS	58	42
Spleen	+	75	LN	+	25	LPS	82	18
Spleen	+	50	LN	+	50	LPS	77	23
Spleen	+	25	LN	+	75	LPS	55	45
Spleen	−	50	Spleen	−	50	Con A	53	47
Spleen	−	50	LN	−	50	Con A	42	58
Spleen	+	50	Spleen	−	50	Con A	0	100
Spleen	−	50	LN	+	50	Con A	86	14

The cells were mixed in various combinations with each other in proportions based on the viable cell count for culture with LPS or Con A, and karyotype analysis was performed after 3 days.

* Results taken from Fig. 2b for LPS-stimulated spleen and lymph node mixtures, neither cell type having been treated with anti-θ serum prior to culture.

was mixed with anti-θ-treated or untreated chromosomally distinct spleen cells in proportions based on the viable cell counts of both populations, the relative response of the lymph node cell population to LPS should be increased. However, it can be seen in Table 1 that an anti-θ treated lymph node cell population mixed with similarly treated, or untreated, spleen cells in proportions based on viable (trypan blue excluding) cell counts, had apparently no more LPS-responsive cells than the untreated lymph node cells studied previously (Fig. 2b).

DISCUSSION

The purpose of this study was to explore further the potential of a chromosome marker technique for assaying mitogen responsiveness of mouse lymphocytes *in vitro*. Previously, a direct comparison of the cytological technique with quantification of responsiveness by means of radioactive thymidine uptake suggested that the magnitude of the response observed with the former method may not be as susceptible to variations in culture conditions (for example, the density of the cultured cells (Watkins and Moorhead, 1969)) as was the degree of tritiated thymidine utilization (Doenhoff, 1971). The original experiments were concerned only with blood cells responding to PHA, this mitogen having been shown to be relatively specific in stimulating blood-borne T cells to divide (Doenhoff *et al.*, 1970). It was hoped that by incorporating spleen cells (the T cells of which are also specifically stimulated to divide by both PHA and Con A (Doenhoff *et al.*, 1974)) in the cultures, the method could be adapted for quantification of B cells responding by mitosis to LPS stimulation (Gery *et al.*, 1972; Doenhoff *et al.*, 1974). It has been assumed that these mitogens act as specifically when stimulating T and B cells in lymph nodes and Peyer's patches as when activating spleen and blood lymphocytes.

Spleen cells from T6T6 mice were generally used throughout as the standard for comparing the PHA, Con A and LPS responsiveness of lymphoid tissues of Lac mice, and in Table 2 the results for mitogen responsiveness of five lymphoid tissues have been tabulated with those observed in current studies for the proportion of cells killed by cytotoxic antisera. For ease of comparison of the serological results with those for mitogen responsiveness, both series have been converted to fractions or multiples of the values obtained for spleen.

The proportion of θ-positive and anti-immunoglobulin sensitive cells in the various tissues correlate with those given in Greaves *et al.* (1973) and when the proportion of cells susceptible to anti-θ

Table 2. A comparison of the relative PHA and LPS responsiveness of the cells in lymphoid tissues of untreated Lac mice, with the proportion of cells killed in each of these tissues (but in a different series of Lac mice) by anti-θ antiserum or rabbit anti-mouse immunoglobulin antiserum (Anti-Ig)

	T cells			
Lac tissue	A PHA (per cent)	B Relative to normal T6T6 spleen	C Anti-θ cytotoxicity	D Relative to spleen
Spleen	50 (5)	1	35 (10)	1
Lymph node	68 (8)	2·12*	71 (4)	2·03†
Blood	62 (8)	1·63	59 (3)	1·69
Peyer's patches	35 (5)	0·54	15 (1)	0·43
Thoracic duct	67 (4)	2·03	80 (1)	2·28
	B cells			
	A LPS (per cent)	B Relative to normal T6T6 spleen	C Anti-Ig cytotoxicity	D Relative to spleen
Spleen	55 (10)	1·22*	46 (9)	1
Lymph node	28 (11)	0·38	23 (3)	0·50†
Blood	26 (12)	0·35	18 (1)	0·39
Peyer's patches	35 (7)	0·53	33 (1)	0·72
Thoracic duct	5 (4)	0·05	12 (1)	0·26

Results for mitogen responsiveness of Lac spleen, lymph node, blood and Peyer's patches relative to T6T6 spleen (column A) are derived from Figs 1, 2 and 5, and from unpublished experiments. Thoracic duct lymphocytes were assayed on a separate occasion. In column B the results in A are given relative to a value of unity for PHA- or LPS-responsive cells present in T6T6 spleen (this being the tissue used as the standard of comparison for the five Lac tissues, thus * 68÷32 = 2·12; 55÷45 = 1·22, etc.). The proportion of cells killed by the anti-θ and anti-immunoglobulin sera are given in column C as a cytotoxic index, and in D the value for spleen in both instances has been considered as 1, and the results for the other tissues given as a multiple of the value for spleen (e.g. † 71÷35 = 2·03; 23÷46 = 0·50, etc.). The total number of assays is given in brackets in columns A and C.

cytotoxicity and to anti-immunoglobulin cytotoxicity are summated (Table 2), lymph node and thoracic duct cell isolates are seen to consist mainly (>90 per cent) of θ-positive and immunoglobulin-bearing cells. Nucleated spleen and blood cell populations contain approximately 20 per cent of cells of unknown origin, and more than 50 per cent of Peyer's patch cells were not identifiable as either T or B cells. Apart from the fact that some lymphocytes carry neither θ nor immunoglobulin determinants (Raff, 1970; Stobo, Rosenthal and Paul, 1973), it might be expected that spleen and blood cell suspensions obtained in the manner described here will contain a proportion of cells which are not small lymphocytes.

Both anti-θ analysis and PHA responsiveness indicate that thoracic duct lymph and lymph nodes contain proportionately the greatest number of T cells, followed in decreasing order by blood, spleen and Peyer's patches. Some discrepancies are apparent between T-cell mitogen responsiveness and surface membrane analysis; for example according to anti-θ cytotoxicity, thoracic duct lymph would be expected to contain more PHA-responsive cells relative to spleen than was observed. However, although the results given in Fig. 7a and b indicate that the majority of PHA-responsive cells in spleen and lymph node are θ-positive, it is by no means certain that all θ-positive cells in these or other peripheral lymphoid tissues are T-cell mitogen-responsive. Furthermore, PHA-responsive cells in thoracic duct lymph may not respond in exactly the same manner as spleen cells in the culture conditions employed here. It is also interesting to note that according to both surface antigen analysis and cytologically assayed mitogen responsiveness (Table 2), lymph node contains twice as many T cells as spleen, but that according to mitogen responsiveness assayed by radioactive thymidine uptake (Fig. 4a), both tissues contain approximately the same PHA-responsive potential. The reason for this may be that in cultures consisting predominantly of lymph node cells, conditions are no longer optimal, particularly in terms of cell density.

With respect to the relationship of LPS-responsive cells to the cells susceptible to the cytotoxic effects of anti-immunoglobulin serum (Table 2), it can be seen that for both characteristics the greatest number of these cells is found in spleen, followed by Peyer's patches > lymph node > blood > thoracic duct cells. However, fewer cells in the lymph node, blood, Peyer's patches and thoracic duct are responsive to LPS stimulation than would be expected from the proportion of cells susceptible to anti-immunoglobulin cytotoxicity, both parameters being related to the values obtained for spleen.

This result may to some extent be explained by the finding that anti-immunoglobulin serum killed no more than 8 per cent of the cells stimulated into metaphase by LPS (Kerbel and Doenhoff, 1974), and if spleen contains a substantial number of activated B cells, it is possible that these will also not be killed by anti-immunoglobulin serum and complement, thus resulting in an artificially low value for the proportion of spleen cells susceptible to anti-immunoglobulin cytotoxicity.

Table 3. A comparison of the relative PHA and LPS responsiveness of spleen cells from normal or T cell-deprived Lac mice, with the proportion of cells killed by either anti-θ antiserum or anti-immunoglobulin antiserum

	T cells			
	A PHA (per cent)	B Relative to normal T6T6 spleen	C Anti-θ cytotoxicity	D Relative to spleen
Normal spleen	50 (5)	1	35 (10)	1
T cell-deprived spleen	13 (5)	0·15	1 (2)	0·03
	B cells			
	A LPS (per cent)	B Relative to normal T6T6 spleen	C Anti-Ig cytotoxicity	D Relative to spleen
Normal spleen	55 (10)	1·22	46 (9)	1
T cell-deprived spleen	60 (5)	1·50	86 (2)	1·85

Results for mitogen responsiveness and cytotoxic indices are given in the same format as in Table 2.

This argument does not explain why LPS responsiveness in the lymph node is not increased relative to that of spleen by removal of θ-positive cells in the former tissue, and mixing the two cell types on the basis of viable cell counts (Table 1). Furthermore, when T cell-deprived spleen is com-

pared with conventional spleen (Table 3), it is found that although the proportion of cells susceptible to anti-immunoglobulin serum had nearly doubled in the former, the proportion of LPS-responsive cells increased by no more than 20 per cent.

These anomalous results relating to B cells could be due to heterogeneity within this population. In particular it may be that though only 20–30 per cent of spleen cells respond to LPS (as adjudged by the presence of surface immunoglobulin (Janossy *et al.*, 1973)), this tissue still contains a far higher proportion of LPS-responsive cells than other peripheral lymphoid organs. It has also been found that responsiveness of Peyer's patch cells to LPS is dependent on the presence of T cells (Kagnoff, Billings and Cohn, 1974), and the response to pokeweed mitogen of B cells isolated from human tonsil is substantially reduced in the complete absence of T cells (Greaves, Janossy and Doenhoff, 1974b). Although this T-cell dependence of B-cell responsiveness to LPS is not observed in spleen cells from congenitally athymic ('nude') mice (Greaves *et al.*, 1974a; Sher, Strong, Ahmed, Knudsen and Sell, 1973), it would appear that other peripheral lymphoid tissues differ from spleen in their relative content of T cell-dependent LPS-responsive cells.

In summary, quantification of the PHA- and Con A-responsive potential of T cells gives results which correlate well with their enumeration by means of *θ* antigen analysis. However, LPS responsiveness appears to be a property of only a subpopulation of B cells, and this finding highlights the importance of using a variety of both surface marker and functional tests for determining the number of T and B cells in a population of lymphocytes.

ACKNOWLEDGMENTS

The authors are grateful to: Dr D. I. Connell for supplying the germ-free T6T6 mice; Dr T. Sadler for providing thoracic duct lymphocytes; Dr E. Leuchars, Mr K. Gomer and Miss J. Snajdr for technical expertise; Miss M. Butt and Mrs J. Atkin for secretarial assistance; the Chester Beatty Research Institute photographic department for preparation of the illustrations; and Drs A. J. S. Davies and M. F. Greaves for helpful discussion. This work was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the Cancer Research Campaign. M.J.D. held a Medical Research Council Fellowship; G.J. was supported by the Medical Research Council and R.S.K. held a National Cancer Institute of Canada Fellowship.

REFERENCES

Davies A.J.S., Leuchars E., Wallis V. & Koller P.C. (1966) The mitotic response of thymus-derived cells to antigenic stimulus. *Transplantation*, **4**, 438.

Doenhoff M.J. (1971) The effect of X-rays on circulating lymphocytes of mice quantitated by PHA responsiveness. *Clin. exp. Immunol.* **8**, 603.

Doenhoff M.J. & Davies A.J.S. (1971) Reconstitution of the T-cell pool after irradiation of mice. *Cell. Immunol.* **2**, 82.

Doenhoff M.J., Davies A.J.S., Leuchars E. & Wallis V. (1970) The thymus and circulating lymphocytes of mice. *Proc. roy. Soc. B*, **176**, 69.

Doenhoff M.J., Janossy G., Greaves M.F., Gomer K.J, & Snajdr J. (1974) Lymphocyte activation. VI. A re-evaluation of factors affecting the selectivity of polyclonal mitogens for mouse T and B cells. *Clin. exp. Immunol.* **17**, 475.

Festenstein H. (1968) Mouse peripheral blood lymphocytes in culture. *Lancet*, **i**, 182.

Ford C.E. (1966) Appendix I. The use of chromosome markers. *Tissue Grafting and Irradiation* (ed. by H. S. Micklem and J. F. Loutit), p. 197. Academic Press, New York.

Forman J. & Möller G. (1973) The effector cell in antibody-induced cell mediated immunity. *Transplant. Rev.* **17**, 108.

Gery I., Krüger J. & Spiesel S.Z. (1972) Stimulation of B lymphocytes by endotoxin. *J. Immunol.* **108**, 1088.

Gorczynski R.M. & Rittenberg M.V. (1974) Stimulation of early protein synthesis as an assay of immune reactivity: an analysis of the cells responding to mitogens and alloantigens. *J. Immunol.* **112**, 47.

Greaves M.F. & Janossy G. (1972) Elucidation of selective T and B lymphocyte responses by cell surface binding ligands. *Transplant. Rev.* **11**, 87.

Greaves M.F., Janossy G., Feldmann M. & Doenhoff M. (1974a) Polyclonal mitogens and the nature of B lymphocyte activation mechanisms. *Genes Receptors Signals* (ed. by A. Williamson and E. Sercarz), p. 271. Academic Press, New York.

Greaves M.F., Janossy G. & Doenhoff M. (1974b) Selective triggering of human T and B lymphocytes *in vitro* by polyclonal mitogens. *J. exp. Med.* **140**, 1.

Greaves M.F., Owen J.J.T. & Raff, M.C. (1973) *T and B Lymphocytes: Origins, Properties and Roles in Immune Responses*, p. 77. Excerpta Medica, Amsterdam and American Elsevier, New York.

Janossy G., Greaves M.F., Doenhoff M.J. & Snajdr J. (1973) Lymphocyte activation. V. Quantitation of the

proliferative responses to mitogens using defined T and B cell populations. *Clin. exp. Immunol.* **14**, 581.

KAGNOFF M.F., BILLINGS P. & COHN M. (1974) Functional characteristics of Peyer's patch lymphoid cells. II. Lipopolysaccharide is thymus dependent. *J. exp. Med.* **139**, 407.

KERBEL R.S. & DOENHOFF M.J. (1974) Resistance of mitotic B lymphocytes to cytotoxic effects of anti-Ig serum. *Nature (Lond.)*, **250**, 342.

KERBEL R.S., ELLIOTT E.V. & WALLIS V.J. (1974) Assessment of mitotic thymus-derived lymphocytes by their sensitivity to the cytotoxic effects of anti-θ serum. *Cell. Immunol.* **11**, 146.

PARROTT, D.M.V. & FERGUSON A. (1974) Selective migration of lymphocytes within the mouse small intestine. *Immunology*, **26**, 571.

PETRIS S. DE & RAFF M.C. (1972) Distribution of immunoglobulin on the surface of mouse lymphoid cells as determined by immunoferritin electron microscopy. I. Antibody induced, temperature-dependent redistribution and its implications for membrane structure. *Europ. J. Immunol.* **2**, 523.

RAFF M.C. (1970) Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology*, **19**, 637.

RAFF M.C. (1971) Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transplant. Rev.* **6**, 52.

RAFF M.C., STERNBERG M. & TAYLOR R.B. (1970) Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature (Lond.)*, **225**, 553.

SHER I., STRONG D.M., AHMED A., KNUDSEN R.C. & SELL K.W. (1973) Specific murine B cell activation by synthetic single and double-stranded polynucleotides. *J. exp. Med.* **138**, 1545.

STOBO J.D. & PAUL W.E. (1973) Functional heterogeneity of immune lymphoid cells. III. Differential responsiveness of T cells of phytohaemagglutinin and Concanavalin A as a probe for T cell subsets. *J. Immunol.* **110**, 362.

STOBO J.D., ROSENTHAL A.S. & PAUL W.E. (1972) Functional heterogeneity of murine lymphoid cells. I. Responsiveness to and surface binding of Concanavalin A and phytohaemagglutinin. *J. Immunol.* **108**, 1.

STOBO J.D., ROSENTHAL A.S. & PAUL W.E. (1973) Murine lymphocytes without detectable theta or immunoglobulin determinants. *J. exp. Med.* **138**, 71.

WAKSMAN B.H. (1973) The homing pattern of thymus-derived lymphocytes in calf and neonatal mouse Peyer's patches. *J. Immunol.* **111**, 878.

WATKINS S.M. & MOORHEAD J.F. (1969) The effect of cell crowding on the *in vitro* reactivity of normal and abnormal human lymphocytes. *Cell Tissue Kinet.* **2**, 213.