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Characterization of the Blastogenic Response of C58 Spleen Cells: Age-dependent Changes

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Summary. Optimum conditions were determined for the stimulation of DNA synthesis in C58 spleen cells by phytohaemagglutinin and allogeneic BALB spleen cells. Maximum stimulation occurred in 3 days in both cases. The rate of DNA synthesis was constant over a 4-hour labelling period with tritiated thymidine. DNA synthesis was significantly enhanced by 15 mM bicarbonate. The peak response of C58 spleen cells to PHA and allogeneic BALB spleen cells occurred when the mice were 3–7 months old; it decreased thereafter. This finding is discussed in relation to age-dependent immune polioencephalomyelitis in C58 mice.

INTRODUCTION

When C58/wm mice, 9 or more months old, are immunized with formalinized (1:500) syngeneic malignant lymphocytes (line Ib) they develop an autoimmune paralytic central nervous system disease (Murphy, Tam, Lanzi, Abell and Kauffman, 1970). Physical signs develop 9–15 days after immunization and the disease is characterized histopathologically by round cell infiltration of the grey matter of the spinal cord and medulla (Lawton and Murphy, 1973). We have called the disease immune polioencephalomyelitis (IPE) (Sager, Lawton and Murphy, 1973).

The pathogenesis of IPE appears to be a cellular immune response to lymphocytic leukaemia antigen (Sager *et al.*, 1973). Therefore we studied the stimulation of DNA synthesis (blastogenic response) in C58/wm spleen cells as an *in vitro* correlate of cellular immunity (Rodey and Good, 1969; Coifman, Good and Meuwissen, 1971; Phillips, Carpenter & Merrill, 1972a). The objective was to develop a simple *in vitro* assay that would facilitate a detailed analysis of the pathogenesis of IPE and the immune response of normal C58 spleen cells to line Ib lymphocytic leukaemia cells.

This paper reports (i) the characterization of the *in vitro* response of C58/wm spleen cells to phytohaemagglutinin (PHA) and allogeneic BALB/wm spleen cells, and (ii) the changes in responsiveness with age.

MATERIALS AND METHODS

Mice

The origin of our inbred strains of BALB/wm (BALB) and C58/wm mice was reported (Murphy *et al.*, 1970).

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Phytohaemagglutinin

Bacto-phytohaemagglutinin P was obtained from Difco Laboratories, Detroit, Michigan. The full strength stock solution was made up in Hanks' balanced salt solution (HBSS), diluted 1:100 or 1:500, and stored at $-20°$. The optimum dose of PHA for stimulation of DNA synthesis was 0·02 ml of a 1:100 dilution in a final volume of 1 ml of culture containing 2×10^6 cells/ml and 5 per cent calf serum. For routine stimulation 0·1 ml of a 1:500 dilution was added per ml of culture fluid.

Leucocyte growth medium (LGM)

LGM was prepared from Ham's F10 medium without bicarbonate (Grand Island Biological Company, Grand Island, New York). F10 was supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, 5 per cent newborn calf serum (Colorado Serum Company, Denver) and 15 mM $NaHCO_3$. HEPES buffer (Schwartz–Mann, Orangeburg, New York) was added (0·02 M) and the pH adjusted to 7·4 with NaOH.

Spleen cell suspensions

Mice were killed by cervical dislocation, the spleens removed, cut into small pieces and homogenized in a Ten Broeck tissue grinder containing 10 ml of HBSS. Cells were decanted and sedimented by centrifugation (550 ***g*** for 15 minutes). Erythrocytes were lysed by exposure to 0·3 per cent NaCl solution for 20 seconds. Spleen cells were then washed twice in HBSS and resuspended in LGM.

Blockage of DNA synthesis

Spleen cells (10^7/ml of LGM) were exposed to 40 μg/ml of mitomycin C (Sigma Chem. Company, St Louis, Missouri) for 45 minutes at 37°, washed three times in Eagle's minimal essential medium, and resuspended (10^6 cells/ml) in LGM. To test the response of normal C58 spleen cells to allogeneic lymphocytes, C58 spleen cells were cultured with mitomycin-blocked spleen cells obtained from 1–2-month-old BALB mice. In 4-day-old cultures DNA synthesis by C58 spleen cells (2×10^6/ml) was optimally stimulated by $0{\cdot}5 \times 10^6$ mitomycin-blocked BALB cells. Mitomycin-blocked syngeneic C58 spleen cells were added in place of allogeneic cells as a control.

Culture technique

C58 spleen cells (4×10^6/ml) were dispensed (0·5 ml) into 10 × 75 mm polypropylene tubes (Falcon, cat. number 2063). Tubes then received either 0·5 ml of mitomycin-blocked BALB spleen cells (10^6/ml) or 0·1 ml of PHA (1:500), the amounts found to give optimal stimulation. Control cultures contained either blocked C58 cells or no PHA. Culture volumes were made up to 1 ml with LGM and contained 2×10^6 C58 spleen cells per ml. Incubation was for 3 days at 37° in a CO_2 incubator (3 per cent CO_2 + 97 per cent humidified air). All cultures were prepared in triplicate.

Differential and viable cell counts

For differential counts spleen cells were suspended in a minimum amount of serum, air-dried as a film on a glass coverslip, and stained with Wrights' stain. Seventy to 85 per cent of cells were small lymphocytes, <1 per cent were polymorphonuclear cells and the remainder were medium size lymphocytes or large mononuclear cells.

The presence of a small proportion of macrophages ($\geqslant 0{\cdot}5$ per cent) is important in the

proliferative response to allogeneic cells, in both the human and mouse systems (Alter & Bach, 1970; Phillips, Carpenter and Merrill, 1972b). Macrophages were counted as described by Cline and Lehrer (1968); cell suspensions were exposed to a 0·05 per cent suspension of polystyrene particles (1 μm diameter) for 30 minutes at 37°. Large cells that took up one or more particles, or small mononuclear cells that took up three or more particles, were scored as macrophages. Final spleen cell suspensions contained 1–5 per cent macrophages. Cell viability was determined by Trypan Blue dye exclusion. Initial viability ranged from 80–98 per cent and was 45–70 per cent after 3–4 days in culture.

DNA synthesis

DNA synthesis was measured by the incorporation of tritiated thymidine ($[^3H]TdR$) into acid-precipitable material. Cultures were pulse-labelled for 4 hours at 37° with 2 μCi/ml of $[^3H]TdR$ (New England Nuclear, Boston, Massachusetts; 6·7 Ci/mM) in the presence of additional unlabelled thymidine at a concentration (1 μg/ml) that approximated flooding conditions (Bain, 1970) but did not inhibit DNA synthesis (Doida & Okada, 1967). For assay, a 0·2-ml sample of each culture was diluted in 4 ml of ice cold 0·85 per cent NaCl solution (saline) and filtered through a cellulose acetate filter (0·45 μm pore, 25 mm diam; Millipore Corporation, Bedford, Massachusetts). The filter was washed consecutively with 20 ml of ice-cold saline, 15 ml of ice-cold 5 per cent trichloracetic acid (TCA), and 4 ml of ethanol. Saline and TCA solutions contained 100 μg/ml of unlabelled thymidine. The filter was dried, placed in scintillation fluid (0·01 per cent POPOP and 0·4 per cent PPO in toluene) and counted. The described procedure gave a minimum background, i.e. the count obtained from a zero time sample taken immediately after isotope was added to the culture. In each experiment it was the mean of determinations on two triplicate sets of cultures. Mean counts per min (cpm) were calculated for each group of three replicate cultures, backgrounds subtracted and adjusted to net mean cpm/10^6 spleen cells in the original culture. Stimulation by PHA or allogeneic spleen cells was expressed as the net increase in cpm above the appropriate control value.

RESULTS

CULTURE CONDITIONS

Maximum viability (45–70 per cent) was obtained over a 4-day culture period by the use of a 5 per cent concentration of newborn calf serum. Because serum lots differed widely a single lot of optimum growth quality was used throughout the study. HEPES buffer (0·02 M) was not toxic to C58 spleen cells. In combination with the bicarbonate–CO_2 buffer system it maintained a pH of 7·3–7·4 during the 4-day culture period. When $NaHCO_3$ was added to culture medium DNA synthesis was enhanced (Table 1). RPMI-1640 culture medium (Flow Laboratories, Rockville, Maryland) was not superior to Hams F10 for DNA synthesis.

KINETICS OF DNA SYNTHESIS

Fig. 1 shows the rates of DNA synthesis over a 6-day period in stimulated cultures. The maximum rate occurred in 3 days in both PHA- and allogeneic spleen cell-stimulated cultures. In cultures of C58 spleen cells without added PHA or BALB spleen cells, the rate of DNA synthesis (data not shown) continued to increase up to 6 days. This could have

TABLE 1

EFFECT OF BICARBONATE ON DNA SYNTHESIS BY C58 SPLEEN CELLS

Percentage CO_2*	$NaHCO_3$ (mM)	Net mean cpm/10^6 spleen cells†	
		Unstimulated	PHA-stimulated
0	0	117	364
3	15	225	853
5	22	205	877

* Percentage CO_2 to air in a CO_2 incubator; pH 7·4 was maintained with 0·02 M HEPES buffer with the appropriate CO_2 concentration.

† Incorporation of [^{3}H]thymidine into DNA in 3-day-old cultures prepared from spleens of 4-month-old mice.

resulted from a mitogenic effect of a medium component (Howe & Battisto, 1971), or the C-type oncogenic RNA virus known to be present in C58 spleen cells (Swarzendruber, Ma & Murphy, 1967). Fig. 2 shows that the rate of DNA synthesis by C58 spleen cells was linear over a 4-hour period then 3-day-old cultures (with or without PHA stimulation) were used. Fig. 2 also shows the enhancing effect of 15 mM bicarbonate on the rate of DNA synthesis.

AGE EFFECTS

Fig. 3 shows the response of C58 spleen cells (obtained from 1–16-month-old mice) to PHA or allogeneic, mitomycin-blocked BALB spleen cells. The response to PHA was

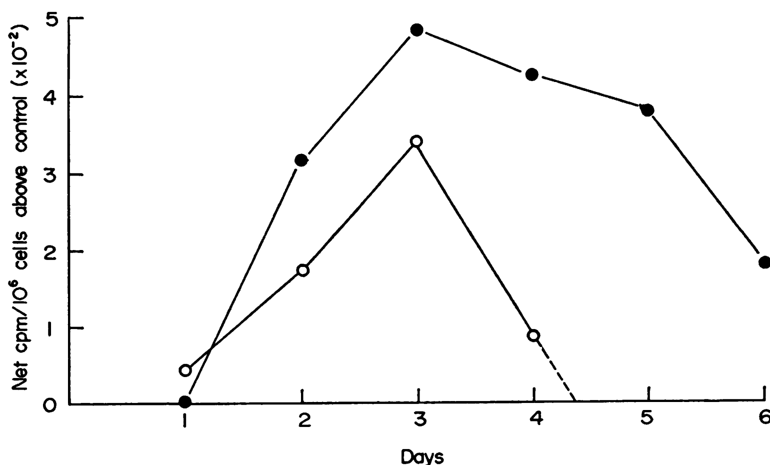

FIG. 1. Rates of DNA synthesis by C58 spleen cells exposed to (●) mitomycin-blocked BALB spleen cells or (○) PHA (1:5000) over a 6-day period. Each point is the mean of three replicate cultures. PHA stimulation was low because bicarbonate was not added to growth medium.

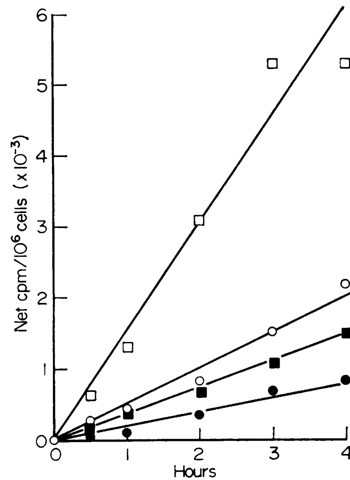

FIG. 2. Incorporation of [^{3}H]TdR into DNA of C58 spleen cells in 68–72-hour cultures. Two-millilitre cultures contained $2–2{\cdot}5 \times 10^6$ cells/ml. [^{3}H]TdR (4 μCi/ml) was added at time 0, 0·1 ml samples assayed at the times indicated and corrected for 0 time background: (●) 3-month-old cells not stimulated with PHA; medium free of added bicarbonate; (○) the same, except that PHA was added; (■) 4-month-old cells not stimulated with PHA but containing 15 mM bicarbonate; (□) the same, except that PHA was added. Data are representative of three separate experiments.

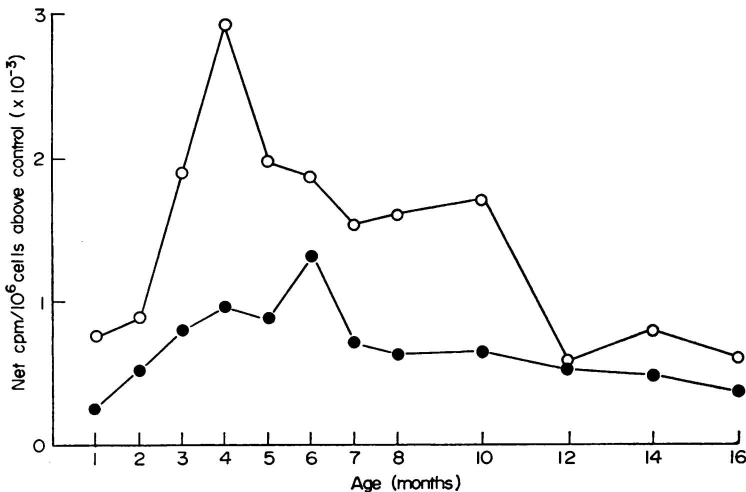

FIG. 3. Effect of age on response of C58 spleen cells to (○) PHA diluted 1:5000 or (●) 5×10^5 mytomycin-blocked BALB spleen cells. The curves show net stimulation above appropriate controls. Each point is the mean of three replicate cultures; experiments were repeated twice to confirm key points.

maximum with spleen cells from 3–6-month-old mice. C58 spleen cells from 3–7-month-old mice gave the maximum response to allogeneic BALB cells.

DISCUSSION

The blastogenic response of lymphocytes *in vitro* as measured by the increase of DNA synthesis is a useful correlate of cellular immunity. There are a number of important

controllable variables influencing the rate of DNA synthesis, such as serum concentration and cell density (Fowler, Hellman, Steinman and Quatrale, 1971), pH and the buffer system (Darzynkiewicz & Jacobson, 1971), etc. Because supplementary serum has mitogenic activity (Howe & Battisto, 1971), we found it best to use the least amount of serum (5 per cent) consistent with good viability. Changes in pH were minimized by using HEPES buffer in combination with bicarbonate. With this combination the pH was maintained at 7·3–7·4 at a cell density of 2×10^6 cells/ml. HEPES buffer up to 0·04 M (Shipman, 1969; Eagle, 1971) is not toxic to a variety of mammalian cells and was not toxic in our culture system. Bicarbonate not only helped to maintain a stable pH but also significantly increased the rate of DNA synthesis in control and PHA-stimulated cultures.

The validity of using a fixed time assay (4 hours after adding [^{3}H]TdR) to measure the rate of DNA synthesis was established by obtaining a zero time assay in each experiment to give the true background counts for the method and also by showing that the rate of incorporation of the labelled nucleoside was constant over the 4-hour period. The dose of PHA that gave optimal stimulation of C58 spleen cells was very close to that reported by Lindahl-Magnusson, Leary and Gresser (1972). We observed the usual sharp dose–response of spleen cells to PHA but also found that PHA solutions lost activity when stored more than 2 weeks at 4°. More consistent results were obtained using allogeneic stimulation, and the latter is thought to be a better indicator of T-cell (thymus-dependent) activity (Coifman *et al.*, 1971).

The responsiveness to both PHA and allogeneic cells depended on the age of the test mice. Peak responsiveness was at 4–7 months. These results are in accord with the findings of Adler, Takiguchi and Smith (1971) who reported a similar phenomenon in C57/Bl and CBA strains, which are prone to autoimmune disease, and correlates with the diminished number of precursor antibody-forming cells in both spleen and bone marrow of ageing C57/Bl mice (Kishimoto and Yamamura, 1971). The loss in reactivity of C58 spleen cells to PHA or allogeneic cells after 6 months of age corresponds to the loss in capacity of C58 mice to be immunized to malignant lymphocytes (Murphy *et al.*, 1970), and to their susceptibility to immune polio-encephalomyelitis (IPE) following immunization. Thus the age-dependence of IPE seems to result from the decline in immune capacity with ageing. This is supported by the recent finding that young C58 mice can be made susceptible to IPE by immunosuppression with X-irradiation or cyclophosphamide (Martinez & Sager, unpublished data). We have proposed that IPE results from the breakage of tolerance to an antigen (or antigens) shared by the malignant lymphoid cells and the CNS (Sager *et al.*, 1973). In this model decline in immune capacity favours induction of disease, as in experimental autoimmune thyroiditis in rabbits, where breakage of tolerance to thyroglobulin is facilitated by immunosuppression (Weigle, 1971).

IPE bears a striking similarity to the myelitis and bulbar encephalitis associated with human carcinoma and Hodgkin's disease (Lawton and Murphy, 1973) and it appears to be a useful model for the study of CNS diseases of this kind. Moreover, it demonstrates the general principle of impaired immunological competence predisposing to autoimmune disease.

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