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Differentiation of human peripheral blood B lymphocytes

Y. S. CHOI & R. A. GOOD *Memorial Sloan-Kettering Cancer Center, Walker Laboratory,* 145 *Boston Post Road, Rye, New York* 10580, *U.S.A.*

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Summary. Human B-lymphocyte differentiation was studied by measuring the capacity of such cells, isolated from peripheral blood, to synthesize and secrete Ig after pokeweed stimulation. Results show that a maximum incorporation of [^{3}H]-thymidine took place 2 days before the appearance of detectable Ig-secreting cells. On the 7th day after pokeweed stimulation, when Ig synthesis and secretion are at a maximum, [^{3}H]-thymidine uptake was low. Since inhibition of DNA synthesis 3 days after pokeweed stimulation completely prevents the generation of Ig-secreting plasma cells, initial DNA synthesis is apparently essential before Ig-secreting plasma cells can develop in response to pokeweed stimulation.

INTRODUCTION

Synthesis of immunoglobulin (Ig) by murine B lymphocytes* in response to bacterial lipopolysaccharides (LPS) has been extensively studied at the molecular level (Melchers & Anderson, 1973). Similar studies of human B lymphocytes have not been undertaken because of technical difficulties in radioimmunoassay and the lack of suitable mitogens specific to human B lymphocytes.

Pokeweed mitogen (PWM) has been known to stimulate both B and T lymphocytes to proliferate *in vitro* (Greaves & Janossy, 1972; Waxdal & Basham, 1974). In both mouse (Waxdal & Basham, 1974) and man (Chessin, Börgeson, Welsh, Douglas & Cooper, 1966; Douglas, 1972; Wu, Lawton, Greaves & Cooper, 1972), treatment with PWM increased DNA synthesis as well as the number of intracellular immunoglobulin (Ig)-staining cells in cultures of lymphocytes (Wu, Lawton & Cooper, 1973; Janossy & Greaves, 1975). At the same time, Ig synthesis and secretion were also greatly increased following PWM stimulation (Parkhouse, Janossy & Greaves, 1972; Wu *et al.*, 1973; Waldmann, Broder, Blaese, Durm, Blackman & Strober, 1974). However, the precise relationship between DNA synthesis and the development of Ig-secreting plasma cells, which takes place during *in vitro* cultures with PWM, is not yet clear.

In previous studies, the methods used to enumerate B lymphocytes were to stain with fluorescent anti-Ig (Wu *et al.*, 1972; Janossy & Greaves, 1975), or to use radioimmunoassay to measure secreted Ig (Waldmann *et al.*, 1974; Janossy & Greaves, 1975). We used a biosynthetic method using radioactively labelled amino acids and an improved, reproducible, serological method to quantify both intracellular and secreted radiolabelled Ig (Parkhouse *et al.*, 1972; Choi, Biggar & Good, 1972; Basham &

Correspondence: Dr Y. S. Choi, Memorial Sloan-Kettering Cancer Center, Walker Laboratory, 145 Boston Post Road, Rye, New York 10580, U.S.A.

* Abbreviations used in this paper: B, bursa (or bone marrow)-derived; FCS, foetal calf serum; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharides; MLR, mixed lymphocyte reaction; NP-40, Nonidet-P 40; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PWM, pokeweed mitogen; T, thymus-derived; TCA, trichloroacetic acid; Tdr, thymidine.

Waxdal, 1975). The capacity of peripheral blood B lymphocytes to synthesize and secrete Ig after PWM stimulation was studied in this way. The experimental approach described in this paper could not only be useful for diagnostic purposes in immunodeficiency diseases but also to study a block in human B-lymphocyte differentiation in molecular and cellular terms.

In this paper, it is shown that a maximum DNA synthetic response takes place before the appearance of detectable Ig-secreting cells; proliferation of lymphocytes is then followed by the maturation of B lymphocytes into plasma cells which actively secrete Ig. Maturation of plasma cell precursors appears to be independent of cell division.

MATERIALS AND METHODS

Lymphocytes

Lymphocytes were prepared from freshly drawn venous blood by Ficoll–Hypaque gradient centrifugation (Thorsby & Bratlie, 1970) and washed twice with RPMI 1640 (Grand Island Biol. Co., N.Y.) containing 10% foetal calf serum (FCS).

Pokeweed mitogen

Ten μl of freshly reconstituted PWM (Grand Island Biol. Co., N.Y., Control No. A251515) was added to 1 ml of culture containing 10^6 lymphocytes. This dosage of PWM was used throughout the experiments.

Culture conditions

Lymphocytes were cultured in round-bottom culture tubes (Falcon, Cat. No. 1950) at an initial cell concentration of 5×10^6 cells/5 ml/tube in RPMI 1640 containing 20% decomplemented FCS. The culture tubes were incubated at an angle of 30° at 37° in a humidified atmosphere of 5% CO_2 in air. At the end of the culture, the cells were spun down and suspended at a concentration of 5×10^6 cells/ml in Dulbecco's modified Eagle's medium lacking leucine but containing 5% FCS and 20 μCi/ml L-4,5-[^{3}H]leucine (40 Ci/mmole, New England Nuclear) for [^{3}H]-leucine incorporation (Choi *et al.*, 1972). Incubations were performed in a humidified tissue-culture incubator at 37° in 15% carbon dioxide and 85% air. After the cultures had been incubated for 4 h, the mixture was chilled quickly and centrifuged at 3000 *g* for 10 min to separate the cells from the supernatant. The cell pellet was suspended in 0·05 M Tris-hydrochloride, pH 7·6, at 4° with 0·025 M potassium chloride and 0·005 M magnesium chloride, and lysed by adding Nonidet-P 40 (NP-40) (Shell Chemical Co., N.Y.) to a final concentration of 0·5%. The nuclei and ribosomes were removed by centrifugation at 150,000 *g* for 60 min at 0°. Both the cell lysate and supernatant fractions were subjected to serological assay and trichloroacetic acid (TCA) precipitation to determine total radioactivity.

Culture conditions for DNA synthetic response

The culture vessels used were microtitration plates with flat-bottomed wells (Falcon, Cat. No. 3040), the initial cell number per well being 2×10^5 cells/0·2 ml. The cell suspensions and mitogens were distributed by using Eppendorf pipettes, after which the plates were covered with lids and cultured, as described above. Cultures were set up in triplicate. At the end of the culture, 0·5 μCi [^{3}H]-thymidine (Tdr) (20 Ci/mmole, New England Nuclear) in 5 μl media were pulsed for 16 h. Whole culture mixtures were harvested on a glass filter by a microharvester (Otto Hiller Co., Madison, Wis.), precipitated with 10% TCA, dissolved in 0·5 ml 0·2 N KOH and the radioactivity determined in 5 ml Toluene-Biosolve (Beckman Instrument Co.) by a liquid scintillation counter.

Serological precipitation

It is well known that a serious technical problem in the quantification of Ig in certain radiolabelled cell lysates by serological precipitation with specific anti-Ig serum is the high 'background' radioactivity precipitable by non-specific antisera (Melchers & Andersson, 1973). This problem has apparently not been encountered in similar experiments with myeloma cells or with lymphoblast culture cells (Choi, Knopf & Lennox, 1971; Choi & Good, 1975). We have investigated this problem and have described elsewhere (Choi, 1977) a reproducible serological method to quantify radiolabelled Ig synthesized and secreted by human peripheral blood lymphocytes (PBL). In this study, we used a direct serological precipitation (co-precipitation) method with rabbit antihuman $F(ab)_2$ and purified human IgG. Antiserum to human $F(ab)_2$ was prepared by injecting rabbits with human IgG-$F(ab)_2$. Human IgG-$F(ab)_2$ used as antigen was prepared by the method of Edelman *et al.* (Edelman, Heremans,

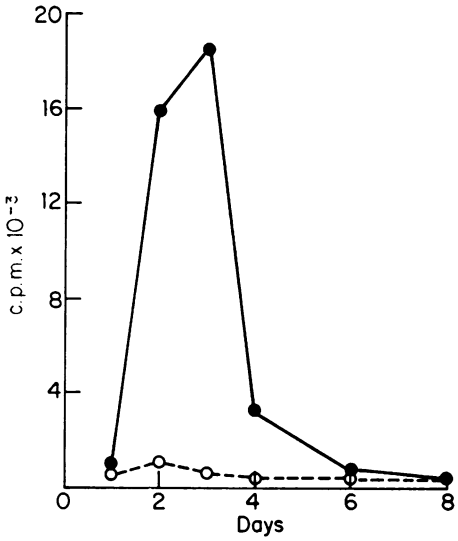

Figure 1. Induction of DNA synthesis by PWM. Microcultures, 2×10^5 cells/0·2 ml/well, were performed in the presence or absence of PWM. Every day after stimulation, 0·5 μCi ^{3}H-Tdr was added for 16 h. (●) PWM added; (○) PWM not added.

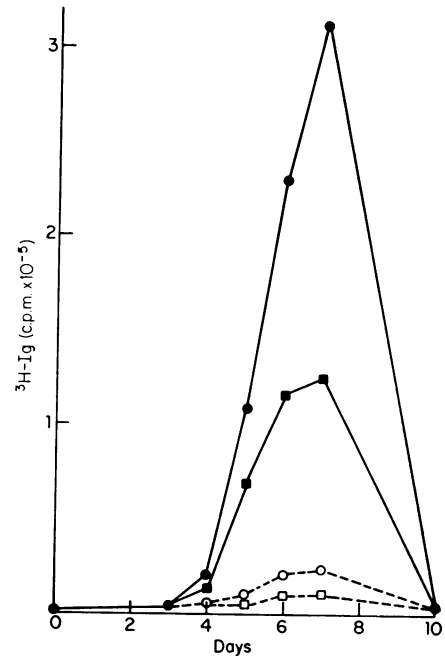

Figure 2. Rates of biosynthesis and secretion of Ig by PBL after PWM stimulation. PBL were cultured, 5×10^6 cells/5 ml/tube, in the presence or absence of PWM. At various dates after stimulation, the cells were spun down and subjected to the short-term (4 h) cultures for [^{3}H]-leucine labelling. Each point shows the total amount of radiolabelled-Ig synthesized (■, □) and secreted (●, ○) by 10^7 cells. (Closed symbol) cultures with PWM; (open symbol) cultures without PWM.

Heremans & Kunkel, 1960). The specificity of this serological assay was carefully examined and reported elsewhere (Choi, 1977).

RESULTS

Induction of DNA synthesis by PWM

In PBL cultured in the presence of PWM and 20% FCS, incorporation of [^{3}H]-Tdr into cellular DNA began to increase 24 h after PWM stimulation and reached a peak after 3 days (Fig. 1), when the rate rapidly decreased, returning to a low level after a further 2 days. In the absence of PWM, the incorporation of [^{3}H]-Tdr remained at a low level during 8–10 days of the culture period. The time of peak incorporation into DNA is earlier than previously reported (6–7 days) (Douglas, 1972; Wu *et al.*, 1972). In the culture conditions described, a peak response was invariably observed 3–4 days after PWM stimulation. However, variations can occur when the sources of lymphocytes, culture media and concentrations of FCS are varied.

Induction of Ig synthesis and secretion

Ig synthesis and secretion did not begin to increase until day 3–4 (Fig. 2). Thus, during the initial 3 days of culture when the rate of Tdr incorporation reached a peak in the presence of PWM, there was little change in the rate of Ig synthesis and secretion. However, from the 4th day on, the rate of Ig synthesis and secretion increased in a linear fashion. The rate of Ig synthesis and secretion by B lymphocytes, when PBL cultures were pulsed with [^{3}H]-leucine, was measured as the amount of [^{3}H]-leucine incorporated into Ig serologically detected in intracellular lysates and in extracellular culture media.

As shown previously (Choi & Good, 1975), intracellular pools of Ig-synthesizing cells become saturated with [^{3}H]-Ig during 4 h of labelling with [^{3}H]-leucine. Hence, the amount of intracellular [^{3}H]-Ig is an indirect measure of the relative number of Ig-synthesizing cells, while the amount of [^{3}H]-Ig

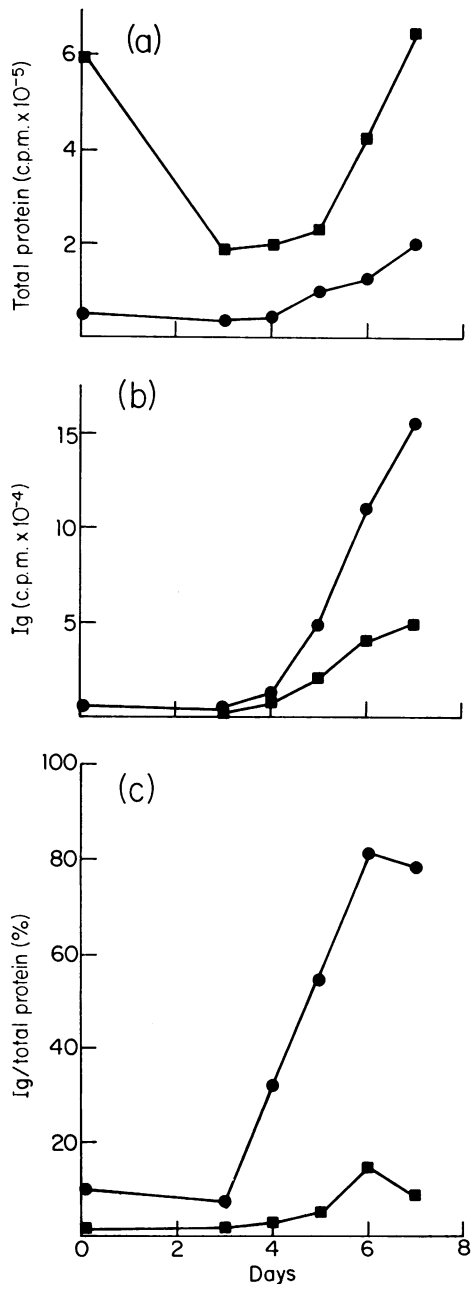

Figure 3. Selective synthesis and secretion of Ig by PBL after PWM stimulation. The experiments were performed in the same manner as described in Fig. 2. Each point shows radiolabelled proteins synthesized (■) or secreted (●) by 10^7 cells. (a) Total protein; (b) Ig; (c) Ig/TCA ratio.

secreted is an estimate of the rate of Ig secretion attributable mostly to plasma cells. An increase of Ig biosynthesis after PWM stimulation is consistent with the increased number of plasma cells which have been observed by the lymphocytes stained for cytoplasmic Ig, as previously reported (Greaves & Janossy, 1972; Wu *et al.*, 1973).

With PWM, the rate of intracellular Ig synthesis and secretion reaches a peak on the 7th day. Without PWM, there was a much less noticeable increase of Ig synthesis and secretion, though there was still a definite peak of Ig synthesis and secretion after culture initiation on days 6–7.

In contrast to these results with human PBL, the peak response of Ig synthesis by mouse spleen cells has been reported to be 3–4 days after PWM stimulation (Basham & Waxdal, 1975).

Selective synthesis and secretion of Ig over other cellular proteins after PWM stimulation

In a separate experiment, protein synthesis and secretion were studied by measuring radioactivity incorporated into TCA-precipitable material. As shown in Fig. 3a, the rate of intracellular protein synthesis decreased rapidly during the first 3 days, but then increased gradually in the same manner as Ig synthesis (Fig. 3b). However, the rates of protein secretion remained low during the first 3 days, but increased gradually from the 4th day on. As shown in Fig. 3c, the Ig/total TCA-precipitable protein ratio of secreted proteins increased from 10% at the beginning to 80% on the 6th day. An eventual Ig/TCA ratio of 80% suggests that virtually all the radioactive proteins secreted by PBL on the 6th day after PWM stimulation are indeed Ig and that the process of B lymphocyte maturation involves selective (preferential) synthesis and secretion of Ig over all other cellular proteins, which reaches a peak 6–7 days after PWM stimulation. This result resembles the maturation process found in mouse B lymphocytes following LPS stimulation (Melchers & Andersson, 1974).

Inhibition of DNA synthesis by [^{3}H]-Tdr 'hot pulse'

As shown by [^{3}H]-Tdr incorporation, cellular proliferation reaches a maximum 3–4 days before the appearance of the maximum number of Ig-secreting cells (Figs 1 and 2). However, the precise relationship of cellular proliferation to the differentiation of

Table 1. Effects of ^{3}H-Tdr pulse on B-lymphocyte development*

Days		Control	Ig (c.p.m. × 10^{-3}) 10^7 cells† ^{3}H-Tdr pulse + block	Inhibition (%)	^{3}H-Tdr pulse	Inhibition (%)
3	I	69·9	49·6	30	1·3	98
	S	129·4	77·4	40	1·8	99
4	I	66·3	52·8	20	4·7	93
	S	138·5	95·1	30	7·8	94
5	I	82·4	85·3	0	26·8	68
	S	156·5	163·2	0	46·3	70
6	I	120·8	111·8	7	69·8	42
	S	259·9	237·0	9	173·5	33

* ^{3}H-Tdr, 20 Ci/mM, was added at the concentration of 20 μCi/ml to the cultures (5×10^6 cells/5 ml) on the days shown. [^{3}H]-Tdr pulse was terminated 16 h later by removing the culture medium and then washing. The cultures continued in a fresh medium without PWM.

† All cultures were harvested on day 7 and assayed for their capacity to synthesize and secrete Ig.

B lymphocytes is not clearly understood. Since the experiments for cellular proliferation and Ig synthesis were performed in two different culture systems, we may not assume that the cells behave in an identical manner.

To explore further the relationship between cellular proliferation and Ig synthesis, we have studied the effects of inhibition of DNA synthesis on Ig synthesis in the same cultures. We have used the 'hot pulse' suicide technique of Dutton & Mishell (1967) who used it to show that the *in vitro* antibody responses of mouse spleen cells could be eliminated by exposing them to [^{3}H]-Tdr of high specific activity at an appropriate time.

The following experiment was undertaken to determine the times at which the elimination of proliferating cells would also abrogate the subsequent emergence of Ig-secreting plasma cells: radioactive Tdr of high specific activity (20 Ci/mM), 20 μCi/ml, was added to the cultures at various times after PWM stimulation, and the [^{3}H]-Tdr pulse terminated 16 h later by removing the culture media containing [^{3}H]-Tdr and then washing the cells once with the medium containing 300-fold excess cold Tdr (100 μg/ml). The culture was continued in fresh medium without PWM. Control cultures were of two kinds: the first received both radioactive and excess cold Tdr (100 μg/ml) together at the time of the pulse; a second was treated the same way but received no [^{3}H]-Tdr.

All cultures were harvested on the 7th day and assayed for their capacity to synthesize and secrete Ig using [^{14}C]-amino acids. Table 1 shows that a 'hot pulse' of [^{3}H]-Tdr applied to the cultures on the 3rd day, when DNA synthetic responses reach a peak, almost completely prevented the development of Ig-secreting plasma cells. However, a 'hot pulse' of [^{3}H]-Tdr on the 6th day prevented only 30–40% of B-lymphocyte differentiation, suggesting that the effect of [^{3}H]-Tdr suicide gradually decreases as the time of 'hot pulse' was delayed after 3 days of PWM stimulation. Even though the rate of DNA synthesis is minimal on the 6th day as measured by [^{3}H]-Tdr incorporation (Fig. 1), the effect of [^{3}H]-Tdr on the development of Ig-secreting plasma cells is still remarkable (i.e. 30–40% suppression). The degree of inhibition at each pulse time was very close both in Ig synthesis and secretion, suggesting that [^{3}H]-Tdr suicide indeed prevents the development of plasma cells which synthesize as well as secrete Ig.

A significant inhibition was also observed on day 3–4, in the cultures in which the effect of [^{3}H]-Tdr pulse was supposed to be prevented by the simultaneous addition of excess cold Tdr (100 μg/ml). This inhibition may be attributed to the metabolic effect of high concentrations of Tdr itself (Xeros, 1962; Harris, Walford & Olsen, 1975). This inhibitory effect of Tdr was no longer to be observed on day 5–6.

The majority of the B lymphocytes which differentiate into plasma cells 7 days after PWM stimula-

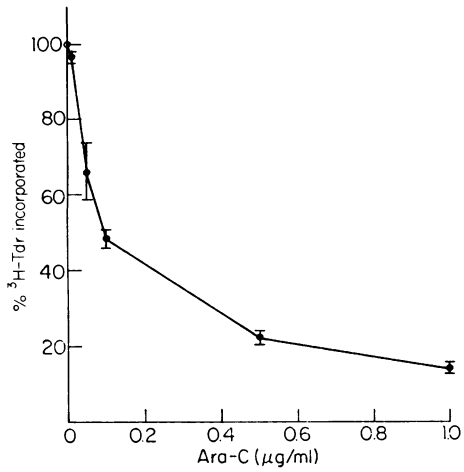

Figure 4. Ara-C inhibition of DNA synthesis after PWM stimulation. Various doses of Ara-C were added with [³H]-Tdr (0·5 μCi/ml) 2 days after PWM stimulation. The amounts of [³H]-Tdr uptake per 2 × 10⁵ cells were measured 16 h later and compared with that of the control culture without Ara-C. Percent inhibition was calculated from this.

tion thus appear to be derived from cells which incorporate Tdr into DNA within 3 days after PWM stimulation. Since [³H]-Tdr suicide can still prevent some B-lymphocyte differentiation 6 days after PWM stimulation, a continuous DNA synthesis of plasma cell precursors appears to be necessary for the emergence of the maximum number of Ig-secreting plasma cells. It is interesting to notice that proliferating B lymphocytes on day 3 after PWM stimulation could not be detected either by staining with fluorescent antihuman Ig or by the Ig biosynthetic method (Siegal, Siegal & Good, 1976).

The effect of Ara-C on Ig synthesis

The results obtained with [³H]-Tdr suicide experiments could be confirmed by a similar experiment using a metabolic inhibitor, Ara-C, which is known to inhibit DNA synthesis specifically (Furth & Cohen, 1967).

Before using Ara-C to examine B-lymphocyte differentiation, the dosage of Ara-C required to inhibit DNA synthesis in this system was estimated. Various doses of Ara-C (0·1–1·0 μg/ml) were applied with [³H]-Tdr 2 days after PWM stimulation and the amount of DNA synthesis measured following 16 h pulse with [³H]-Tdr. As shown in Fig. 4, the incorporation of [³H]-Tdr was depressed by Ara-C in a concentration-dependent manner and 0·5–1·0 μg/ml Ara-C inhibited the DNA synthetic responses to PWM stimulation by more than 80%. Treatment with 0·5–1·0 μg/ml Ara-C for 24 h allowed the recovery of 85–90% of the viable cells. Since the difference in the inhibitory effect between 0·5–1·0 μg/ml was small, 0·5 μg/ml Ara-C was chosen for the experiments on its effect on B-lymphocyte differentiation.

At various times after PWM stimulation, 0·5 μg/ml Ara-C was added for 24 h and the capacity of B lymphocytes to synthesize and secrete Ig assayed after the removal of Ara-C. Untreated control cultures were assayed at the same time for Ig synthesis and secretion.

As shown in the data in Table 2, 0·5 μg/ml on the 3rd day caused an average reduction of 70% Ig synthesis and 50% Ig secretion. As the time of Ara-C addition was delayed, the inhibitory effect of Ara-C decreased; on the 7th day there was only a 20% reduction in Ig synthesis and secretion. We have repeated this experiment on three separate occasions and observed the identical influence of Ara-C.

Addition of Ara-C on the 6th day still inhibited the response to a significant extent (i.e. 37%). Since [³H]-Tdr incorporation is very small on that day, a nonspecific lethal effect of Ara-C may not be ruled out. However, this possibility is less likely because similar experimental results obtained by using [³H]-Tdr suicide techniques clearly showed a gradually decreasing inhibitory effect the longer the culture continued (Table 1).

DISCUSSION

Since PWM stimulates both B and T lymphocytes to proliferate (Greaves & Janossy, 1972; Waxdal & Basham, 1974), the early proliferation as measured by [³H]-Tdr uptake (Fig. 1) can be attributed to a large extent to T lymphocytes in PBL rather than B lymphocytes. As a matter of fact, recently it has been shown that stimulation of B lymphocytes does not take place in the absence of T lymphocytes (Cooper, Keightley & Lawton, 1975; Janossy, Gomez de la Concha, Luquetti, Waxdal & Platts-Mills, 1976), suggesting that PWM may activate T lymphocytes first, which is then followed by B lymphocyte stimulation. Since Ig synthesis and secretion clearly measure only B-lymphocyte differ-

Table 2. Effects of Ara-C on B-lymphocyte development*

Days		Ig (c.p.m. × 10^{-3}) 10^7 cells Control	Ara-C (0·5 μg/ml)	Inhibition (%)
3	I	33·0 ± 2·0	10·1 ± 1·1	70
	S	31·8 ± 2·5	17·3 ± 0·7	46
4	I	93·9 ± 3·1	48·9 ± 2·2	48
	S	138·7 ± 8·9	76·0 ± 0·6	45
5	I	232·9 ± 32·6	135·7 ± 5·5	42
	S	341·0 ± 30·1	233·2 ± 7·8	32
6	I	269·3 ± 10·8	168·8 ± 7·6	37
	S	456·7 ± 13·8	280·3 ± 5·6	39
7	I	304·5 ± 38·2	233·0 ± 22·3	23
	S	538·6 ± 17·9	447·8 ± 14·9	17

* Ara-C, 0·5 μg/ml, was added for 24 h to the cultures on the days shown, and the capacity to synthesize and secrete Ig was assayed the following day. Control cultures were not treated with Ara-C, but Ig synthesis and secretion was assayed at the same time.

entiation, our experimental results describe only cellular events which take place after B cells were stimulated directly or indirectly by PWM.

With human PBL, DNA synthesis is apparently essential before Ig-secreting plasma cells can develop in response to PWM stimulation. This conclusion is based on the following observations: (1) Pulsing cultures with [^{3}H]-Tdr of high specific activity 3 days after PWM stimulation completely prevents the generation of Ig-secreting plasma cells (Table 1); (2) Inhibition of DNA synthesis by Ara-C (0·5 μg/ml), likewise, has a profound suppressive effect on the development of B lymphocytes into plasma cells (Table 2).

The early response of PBL to PWM is thus DNA synthesis. After proliferation, the precursors of plasma cells then enter a phase of maturation during which they acquire the capacity to synthesize as well as secrete Ig. Since the addition of [^{3}H]-Tdr or Ara-C at a late stage of PWM stimulation (day 5–6) was markedly less effective in preventing the emergence of Ig-secreting cells, the maturation of plasma cell precursors (as revealed by the development of Ig-secreting activity) can apparently be independent of cell division.

Recently, Askonas *et al.* (1976) reported that, after stimulation by LPS, Ig synthesis by murine B lymphocytes was inhibited in cultures that have been exposed to Budr at the peak of blast cell production. This result also supports the conclusion that cellular proliferation is linked to the development of plasma cells.

Since Askonas *et al.* (1976) showed that a very small portion of B lymphocytes becomes Ig-secreting plasma cells after LPS stimulation, it is possible that Ig-secreting cells may develop, in response to PWM stimulation, from non-dividing cells. Harris *et al.* (1975) showed that the differentiation of antibody producing cells was inhibited by Tdr at the dose too low to inhibit cell division, indicating that the terminal differentiation of B lymphocytes requires DNA synthesis unrelated to cell division.

Taken together, our studies essentially support the idea that terminal differentiation of human peripheral blood lymphocytes requires DNA synthesis.

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