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Control of B-cell maturation in mice

I. INCREASED B-CELL MATURATION *IN VITRO* BY BONE MARROW PROTECTED DURING WHOLE BODY IRRADIATION

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Summary. The possibility of a homeostatic control on the production of B cells was studied in CBA mice following whole body irradiation (750 rads). Bone marrow cells from femurs shielded from irradiation were taken at 24 h and the number of surface immunoglobulin positive cells assessed with a fluorescence-activated cell sorter after 24 h *in vitro*. The cells from the irradiated shielded mice showed greater absolute number of 'bright' B cells with a high density of surface immunoglobulin (mean increase 60%–100%) than cells from control unirradiated mice. These bright B cells did not incorporate (^{3}H) thymidine *in vitro* and treatment with hydroxyurea (an inhibitor of DNA synthesis) did not prevent their increase. It was concluded that the increased number of bright B cells *in vitro* arose from augmented maturation or differentiation and not from a proliferative process.

Abbreviations: BM, bone marrow; FACS, Fluorescence-activated cell sorter; FCS, Foetal calf serum; goat α-RbIg, goat anti-rabbit immunoglobulin; HU, hydroxyurea; irrad-shielded, irradiated leg shielded mice; LPS, lipopolysaccharide; MEM, minimum essential medium; NRS, normal rabbit serum; rabbit α-MIg, rabbit anti-mouse immunoglobulin; sIg, surface immunoglobulin.

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INTRODUCTION

The bone marrow (BM) of adult mice is the site of continuous rapid production of B cells (Osmond & Nossal, 1974; Ryser & Vassalli, 1974; Owen, Wright, Habu, Raff & Cooper, 1977). Following sublethal whole body irradiation the residual BM cells proliferate and repopulate the lymphoid system (Harris, 1956; Hulse, 1963; Anderson, Olson, Autry, Howarth, Troup & Bartels, 1977). Similarly, reconstitution of lethally irradiated mice with BM leads to the restoration of B cells and antibody production (Nossal, Cunningham, Mitchell & Miller, 1968; Unanue, Grey, Rabellino, Campbell & Schmidtke, 1971; Nossal & Pike, 1973). These findings suggest that there may be homeostatic control on the production of mature B cells and that irradiation, either by destroying B cells or by some other mechanism, gives rise to a stimulus which leads to increased B-cell production. Using whole body irradiation with protection of one limb, several authors have shown accelerated stem cell (CFU-S) differentiation in the shielded BM (Croizat, Frindel & Tubiana, 1976; Gidali & Lajtha, 1972; Lord, Wright & Mori, 1978). Serum from these partially irradiated mice showed an enhanced ability to stimu-

late growth of granulocytic colonies *in vitro* (Morley, Rickard, Howard & Stohlman, 1971). These experiments provide evidence for humoral factors which affect the differentiation of haemopoietic progenitor cells. Using the same experimental model, we have investigated whether the protected BM from whole body irradiated mice showed increased B-cell production. In order to avoid the possible complications arising from migration of B cells at various stages of maturation from the BM to the spleen (Brahim & Osmond, 1970; Osmond & Nossal, 1974) we studied B-cell production *in vitro* by BM protected during whole body irradiation. This paper shows that, under these conditions, there is an increased appearance of B cells with a high density of surface immunoglobulins, which arise by a process which does not require cell division.

MATERIALS AND METHODS

Mice

CBA male mice, 6–12 weeks old, were bred locally at the Clinical Research Centre.

Irradiation

Mice were anaesthetized with intraperitoneal pentobarbital sodium 60 mg/kg and given 750 rads gamma irradiation from a ^{60}Co source at a rate of about 28 rads/min. Mice were placed on a 3 cm thick lead brick and the hind limbs shielded by 10 cm lead bricks and protected from scattered radiation by 3 cm lead bricks at the sides. Protection was validated by showing that irradiation, when the whole animal was shielded, caused no change in the peripheral white blood cells or the bone marrow nucleated cell counts, when compared to normal unirradiated control mice. Control mice were anaesthetized and either left untreated or irradiated while protecting the whole body ('sham irradiated' animals).

Preparation of cell suspensions

Mice were anaesthetized with ether and bled out. BM from femurs and spleen cell suspensions were prepared aseptically, filtered, washed twice with culture medium at 4° and resuspended at 2×10^6 live cells/ml. White cell counts were carried out by electronic counting (Coulter Counter, model DN, Coulter Electronics) after lysing the red blood cells by Zaponin (Coulter Diagnostics). Cell viability was determined by 0·1% trypan blue dye exclusion (final serum concentration 1%).

Culture

RPMI 1640 with sodium bicarbonate (Flow Laboratories), supplemented with 10% inactivated horse serum (Wellcome Research Laboratories, Beckenham), 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2×10^{-5} M 2-mercaptoethanol, was used. 2×10^6 viable cells/ml in 0·25 ml aliquots were placed in 6 mm flat-bottom microtitre plates (Sterilin, Teddington) and cultured at 37° in a humidified atmosphere of 5% CO_2 in air. In the standard experiments, pools of cells from two to three mice were dispersed in five to ten wells and harvested after 2 and 24 h of culture. In some experiments, hydroxyurea (HU) (Sigma) was added to the initial culture (0·3 mg/ml final concentration) to inhibit DNA synthesis (Janossy & Greaves, 1975).

Mitogenic stimulation

Lipopolysaccharide (LPS) from *Escherichia coli* 0127.B8 (Difco Laboratories) was added after 2 or 24 h of culture at a final concentration of 30 μg/ml. The culture continued for a further 2 days.

Tritiated thymidine (^3H) TdR (1 μCi per well; specific activity 200 μCi/mmol) (TRA 120 Radiochemical Centre, Amersham) was added to cultures which were harvested 24 h later in a multiple cell culture harvester (Skatron Automatic Harvester, type MCH 1). Radioactivity was measured using Toluene/PPO/POPOP scintillation fluid in a scintillation spectrometer.

Immunofluorescent studies with fluorescence-activated cell sorter (FACS)

All procedures were carried out at 4° in Eagle's MEM containing sodium azide (1 mg/ml). After cell culture, $2{\cdot}5 \times 10^6$ cells were washed once in 5 ml plastic test tubes by centrifugation for 2 min in an angle-head Serofuge centrifuge, then incubated for 30 min with a rabbit anti-mouse Ig (final dilution 1/64) (anti G1, G2a, G2b, G3, IgA, IgM heavy and light chains, Nordic Immunological Laboratories), or inactivated normal rabbit serum (same dilution) as a control, followed by three washes in Eagle's MEM; they were then incubated 30 min with fluorescein conjugated goat anti-rabbit IgG (Fab + Fc) (Nordic Immunological Laboratories) (final dilution 1/80), followed by three washes. The cells were finally resuspended in 1 ml and analysed in a FACS II (Becton, Dickinson FACS

Systems, Mountain View, Calif.). They were processed at a rate of 1000 cells/s with the laser set at 488 nm, 300 mW and the photomultiplier at 850 V (see Loken & Herzenberg, 1975). Twenty thousand cells were analysed for light scatter (cell size) and fluorescence intensity.

Light scatter profiles. The light scatter profiles of BM cells from normal unirradiated mice and from irrad-shielded mice after 2 h culture were identical (Fig. 1a). The peak on the left hand represented mainly red cells and non-viable cells and was always excluded from the fluorescent profiles used to estimate the percentage of B cells. In fact this light scattering criterion of dead cells detects a similar number of dead cells to the more conventional dye exclusion tests. In one experiment BM cells freed from red cells by lysis in Boyle's solution contained 12%, 15% and 17% dead cells by trypan blue dye exclusion, ethidium bromide dye exclusion and light scatter respectively. In the case of lymph node cells the figures were 33%, 36% and 40%. This makes it likely that the light scattering and the dye exclusion tests for cell death detect the same cells and justifies the use of the dead cell percentage to correct the cell count in forming an estimate of the total number of B cells. In fact, the Coulter Counter detects both live cells and dead cells whose nuclear permeability is normal and hence includes most cells which fail to exclude trypan blue.

The middle peak was composed of lymphocytes and the right-hand peak of larger, mainly non-lymphoid, cells (Lala, Johnson, Battye & Nossal, 1979). After 24 h culture the profiles of BM cells from normal and irrad-shielded mice showed more dead cells and debris on the left-hand peak but the two right-hand peaks persisted.

Fluorescence profiles. At all times the profile was unimodal; after 2 h culture the profiles of BM cells from normal mice and from irrad-shielded mice were similar (Fig. 1b). The total viable nucleated cells were routinely analysed. In fact, when small and larger cells were analysed separately, the percentage of specific sIg positive cells was always much higher in the smaller than in the larger cell population (10%–15% *v.* < 0·7%). The results were expressed as the percentage of cells lying under various portions of the fluorescence curve. Cells in channels 0–25 were arbitrarily considered negative, cells in channels 26–110 'dull' cells and cells in channels 111–255 'bright' cells. The percentages of fluorescent cells were always corrected by subtracting

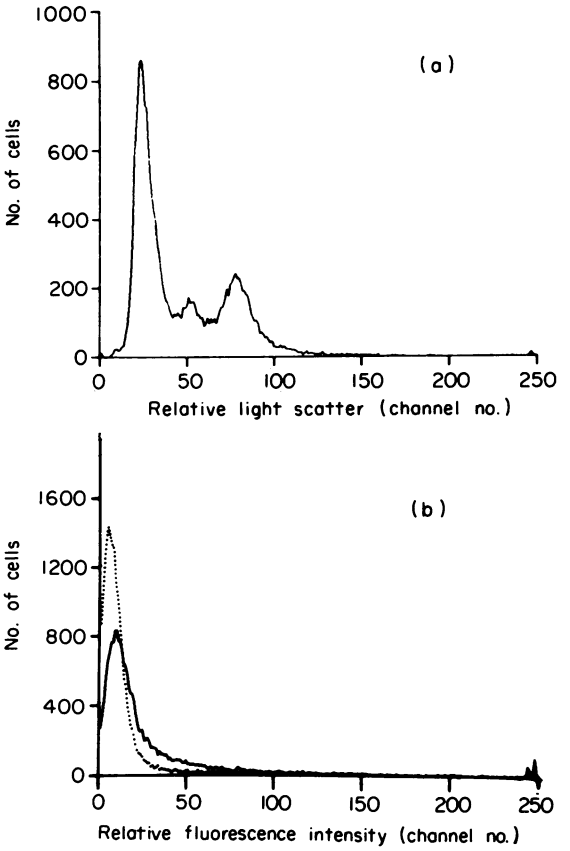

Figure 1. (a) light scatter profile of normal bone marrow cells after 2 h culture. The left-hand peak represents mainly red cells and dead cells and was always excluded for fluorescence measurements. The middle peak represents mainly small lymphocytes and the right-hand peak larger non-lymphoid cells. (b) fluorescence profile of normal bone marrow cells, excluding the left-hand peak of light-scatter profile. The cells were incubated with rabbit α-MIg followed by fluorescent goat α-RbIg (——), and compared with control cells incubated with NRS followed by fluorescent goat α-RbIg (- - -), under non-capping conditions (4°, 0·1% sodium azide).

the percentage of 'control' fluorescence. To exclude macrophages and monocytes which might simulate B cells, positive fluorescent cells were sometimes checked under a microscope for the presence of endogenous peroxidase activity by the method of Preud'homme & Flandrin (1974). No positive fluorescent cells showed endogenous peroxidase activity.

Immunoperoxidase staining and autoradiography

After culture in the presence of (^{3}H)TdR (1 μCi/ml,

specific activity 200 μCi/mmol, TRA 120, Radiochemical Centre, Amersham), $2{\cdot}5 \times 10^6$ cells were washed with Eagle's MEM (without azide) and incubated with rabbit α-MIg (dilution ½) (Nordic Immunological Laboratories) or NRS (control) at 37° for 30 min; the cells were gently resuspended every 5 min. They were then washed three times in the MEM and the pellet finally resuspended in 1 ml and cytocentrifuged at 1000 rpm for 5 min in a Shandon–Elliot cytocentrifuge. The slides were left to dry at least overnight (Stanislavski, Mitard & Stanislavski, 1976). The cells were then treated with 100% methanol containing 0·01% H_2O_2 for 30 min to destroy endogenous peroxidase activity (Streeferk, 1972), dried 5 min under a fan and rehydrated in phosphate-buffered saline (PBS). The cells were covered with 25 μl of peroxidase-labelled goat anti-rabbit Ig (dilution 1/80) (Avrameas & Ternynck, 1971) and incubated in a moist chamber at room temperature for 45 min. Cells were washed twice in PBS and incubated with 3,3′ diaminobenzidinetetrahydrochloric acid (0·2 mg/ml) (Sigma) containing 0·01% H_2O_2 (Graham & Karnovsky, 1966) for 10 min. With this immunoenzymatic technique, about 45% of spleen cells, 5–10% of bone marrow cells and less than 1% of thymocytes were labelled in normal mice. Most sIg positive lymphocytes showed typical capping. The slides were finally rinsed in distilled water, dried and covered with Kodak AR-10 stripping film, exposed for 1 day to 3 weeks at −20°, fixed, rinsed in cold water and dried in air. Four days of exposure time was chosen as the best time since it gave great numbers of heavily labelled non-lymphoid cells and a very low background.

RESULTS

Basic phenomenon

The following experiments concerned the appearance of B cells in cultures of BM cells from unirradiated control and irrad-shielded mice. Mice were irradiated at 750 rads, the BM taken 24 h later and cultured for 2 and 24 h. The percentages and absolute number of B cells (total sIg positive cells) and B cells with a high level of sIg ('bright' cells) and low level of sIg ('dull' cells) were measured, as explained in Table 1. Tables 1

Table 1. Method of calculation used to obtain absolute numbers of bright sIg positive cells in bone marrow. Data from one representative experiment showing a greater absolute number of bright B cells in bone marrow from irrad-shielded than from normal mice after 24 h in culture*

	2 h culture		24 h culture	
	Control unirradiated	Irrad-shielded	Control unirradiated	Irrad-shielded
Total nucleated cells†	$1{\cdot}54 \times 10^6$	$1{\cdot}57 \times 10^6$	$1{\cdot}87 \times 10^6$	$1{\cdot}79 \times 10^6$
Dead cells‡	10·4%	10·9%	12·3%	8·4%
Live nucleated cells	$1{\cdot}38 \times 10^6$	$1{\cdot}40 \times 10^6$	$1{\cdot}64 \times 10^6$	$1{\cdot}64 \times 10^6$
Total sIg positive cells (%)§ (fluorescence channels: 26–255)	15·1%	20·08%	25·7%	27·9%
Total sIg positive cells (absolute number)	$2{\cdot}09 \times 10^5$	$2{\cdot}81 \times 10^5$	$4{\cdot}2 \times 10^5$	$4{\cdot}57 \times 10^5$
Bright sIg positive cells (%) (fluorescence channels: 110–255)	2·17%	2·47%	7·58%	13·5%
Total bright sIg positive cells (absolute number)	$2{\cdot}99 \times 10^4$	$3{\cdot}47 \times 10^4$	$12{\cdot}43 \times 10^4$	$22{\cdot}12 \times 10^4$
Total dull sIg positive cells (fluorescence channels: 26–110)	$1{\cdot}79 \times 10^5$	$2{\cdot}46 \times 10^5$	$2{\cdot}97 \times 10^5$	$2{\cdot}36 \times 10^5$

* All values are expressed per ml culture medium (all cultures started with the same absolute number of viable nucleated cells) and based on pooled cells from three mice.

† Obtained by Coulter counter

‡ Obtained by trypan blue dye exclusion

§ Cells (20,000) were routinely analysed by FACS after labelling with rabbit α-MIg then fluorescent goat α-RbIg. Percentages are expressed after correction for NRS-binding cells (more than 95% of non-specific labelling was weak).

Table 2. Increased ratio of bright/dull B cells in bone marrow from irrad-shielded mice after 24 h culture

	2 h culture			24 h culture		
	Control unirradiated	Irrad-shielded	*P* values†	Control unirradiated	Irrad-shielded	*P* values†
Live nucleated cells‡	150±8·6*	148·3±6·9	NS	156±25·4	168±26·3	NS
Total sIg positive cells§	23·6±2·06	23·6±3·45	NS	38·97±2·19	43·43±3·70	NS
Total bright sIg positive cells§	3·54±0·62	2·78±0·52	NS	10·08±2·57	20·5±4·66	<0·05
Total dull sIg positive cells§	20·03±1·51	20·66±3·10	NS	28·93±2·47	22·93±1·78	<0·01
Bright/dull ratio	0·18±0·02	0·14±0·02	NS	0·35±0·10	0·89±0·22	<0·05

* Values represent means±SD of three experiments, each based on pooled cells from three mice. They are expressed as 10^4 cells/ml culture medium (all cultures started with the same absolute number of viable nucleated cells)

† Obtained by Student's *t* test.

‡ Obtained by Coulter counter and trypan blue dye exclusion.

§ From FACS analysis; for details see Table 1.

and 2 show there was no obvious difference between the experimental and control cells after 2 h culture. This indicated that the difference observed at 24 h culture arise during culture and not *in vivo*. Table 2 shows that, after 24 h culture, a difference was only seen when bright and dull B cells were considered separately, since the total number of B cells rose both in control and experimental cultures. However, whereas in control cultures the rise was due to an increase of dull *and* bright cells, in the experimental cultures the rise was essentially due to an increase of *bright* B cells. In fact, the mean increase of bright B cells in experimental cultures ranged from about 60% to 100% when compared to control cultures. Fluorescence profiles taken at 24 h culture (Fig. 2a) present the same finding graphically and show that the difference between the control and irrad-shielded cultures is only apparent when the bright cells are considered (channels 180–250).

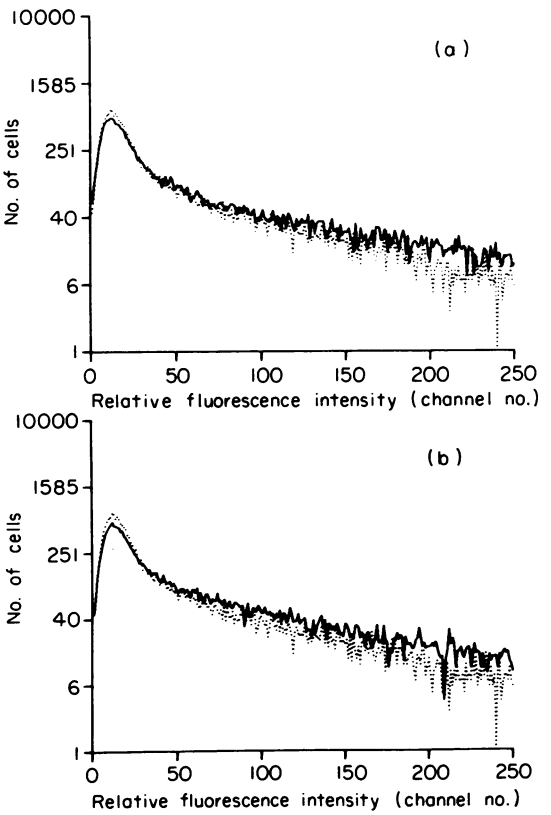

Figure 2. Fluorescence profiles of BM cells incubated with rabbit α-MIg followed by fluorescent goat α-RbIg. (a) normal (- - -) *v.* irrad-shielded (——) cells after 24 h culture. The more intensely fluorescent cells are present in the higher channels. (b) normal (- - -) *v.* hydroxyurea treated (——) cells after 24 h culture. HU cells show a greater proportion of sIg positive cells over a wide range of brightness.

Evidence that increased B cell production in vitro is not due to cell division

Autoradiographic studies. The previous section showed that there was an increased number of bright B cells *in vitro*, after irradiation *in vivo*. However, it is not clear whether cell division *in vitro* was required. The first series of experiments investigated whether BM cells cultured in the presence of (^{3}H) thymidine gave rise to labelled B cells. Cells from control and irrad-shielded mice were cultured in the presence of 1 μCi/ml^{-1} (^{3}H) thymidine. Table 3 and Fig. 3 show that none of the sIg positive cells detected by immuno-per-

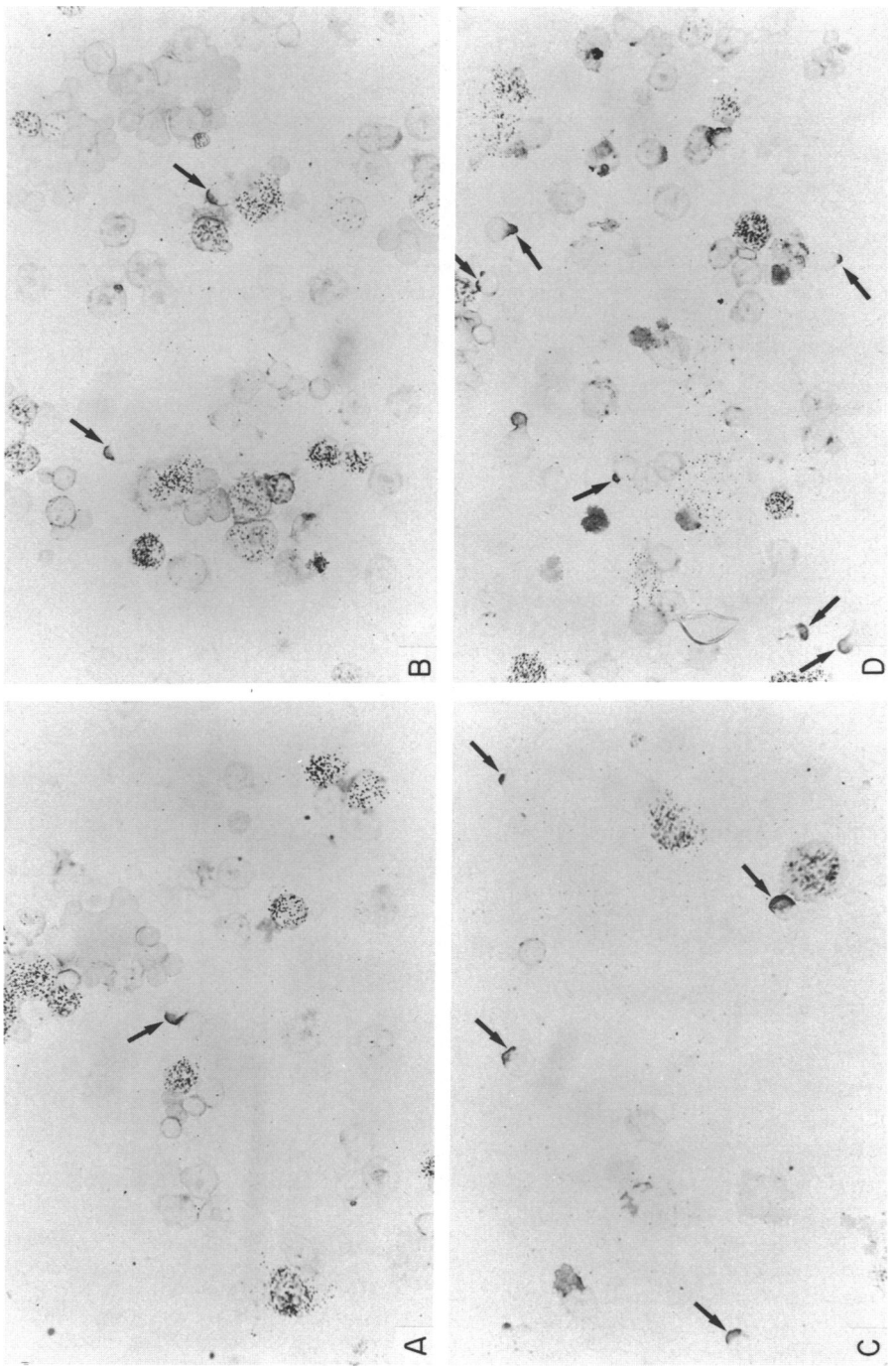

Figure 3. Combined autoradiography and immunoperoxidase (× 440). After culture in presence of (^{3}H) thymidine, sIg positive cells were detected by immunoperoxidase technique. These photographs show that few of the B cells incorporated (^{3}H) thymidine. (a) normal BM cells, 2 h culture. (b) BM cells from irrad-shielded mice, 2 h culture. (c) normal BM cells, 24 h culture. (d) BM cells from irrad-shielded mice, 24 h culture. The arrows point to the B cells.

Table 3. Absence of radioactive sIg positive bone marrow cells after 24 h incubation with tritiated thymidine

Culture time*	Source of BM cells	sIg positive cells†		Other nucleated cells		sIg positve cells (%)
		No.	Radioactive (%) ‡	No.	Radioactive (%) ‡	
2 h	Normal unirradiated	125	0	3230	51	3·72
	Irrad-shielded	144	0	3517	51	3·93
24 h	Normal unirradiated	204	1·9	2494	30	7·56
	Irrad-shielded	561	1·8	3269	26	14·64

* Cells were cultured in microculture plates, each well containing 5×10^5 cells in 0·25 ml of culture medium, in continuous presence of 1 μCi tritiated thymidine. Ten replicates were usually undertaken and pooled at the end of culture.

† sIg positive cells were detected by immunoperoxidase staining (for details, see Materials and Methods).

‡ Autoradiographic exposure, 4 days. Only cells with ten or more overlying silver grains were considered as labelled.

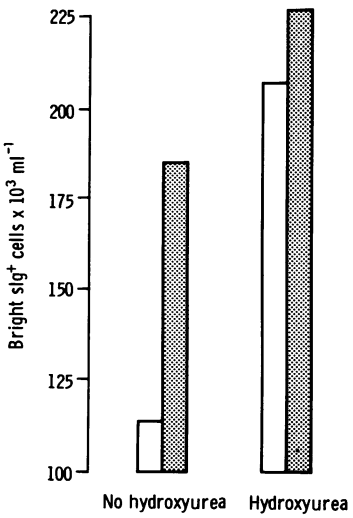

Figure 4. Failure of hydroxyurea to affect production of bright sIg positive cells in BM from irrad-shielded mice after 24 h culture and increase in the number of B cells in normal BM. BM cells were cultured for 24 h in the presence of HU (0·3 mg/ml), stained for sIg and analysed by FACS. Open columns: BM cells from normal, unirradiated mice; filled columns: BM cells from irrad-shielded mice. Data from one representative experiment based on pooled cells of three mice.

Table 4. LPS response of bone marrow cells from normal or irrad-shielded mice (normal spleen = positive control)

Incubation time* (h)	Cells cultured	None	LPS
2	Normal spleen	398 ± 68†	3349 ± 180
	Normal BM	743 ± 38	682 ± 33
	Irrad-shielded BM	1107 ± 111	1011 ± 182
24	Normal spleen	215 ± 22	1575 ± 276
	Normal BM	574 ± 46	421 ± 65
	Irrad-shielded BM	988 ± 104	410 ± 18
48	Normal spleen	145 ± 25	675 ± 83
	Normal BM	282 ± 14	407 ± 87
	Irrad-shielded BM	500 ± 86	478 ± 92

* Cells were cultured in microculture plates, each well containing 5×10^5 cells in 0·25 ml culture medium. LPS (30 μg/ml) was added after 2, 24 or 48 h culture. Responses were measured 2 days later, after a 24 h pulse of 1 μCi titriated thymidine per well.

† Values represent c.p.m. mean ± SD of triplicate cultures.

oxidase had incorporated (^{3}H) thymidine after 2 h culture (Fig. 3a, b) and very few (< 2%) after 24 h of culture (Fig. 3c, d) although many other cells were labelled. It was concluded that most of the bright B cells had not synthesized DNA *in vitro*.

Hydroxyurea studies. Cells were cultured in presence of HU which selectively kills cells in the S phase of the cell cycle (Sinclair, 1967). Despite reduction of DNA synthesis, as shown by autoradiography, HU did not depress the development of bright B cells *in vitro* (Fig. 4), confirming the previous conclusions.

The unexpected observation was made that culture with HU increased the percentage and absolute numbers of B cells (Fig. 4). This was true for both bright and dull B cells, as shown by the fluorescence profiles (Fig. 2b). This experiment was repeated five times with similar results. Other reports have shown the relative number of B cells was increased in marrow cells from mice pretreated with HU (Fuchs, Kaiton, Petrov, Ataullakhanov, Sidorovich, Vanko & Malaitsev, 1978).

LPS stimulation

Attempts were made to investigate the functional capacity of the B cells generated *in vitro* by studying the response to B-cell mitogen LPS (Table 4). Control studies showed that LPS increased DNA synthesis by mouse spleen cells and that this effect was present but less marked when cells were kept *in vitro* for 24 and 48 h before adding LPS. However, LPS depressed DNA synthesis when it was added at 24 h to normal or irrad-shielded BM cells and no conclusion about the mitotic activity of the B cells could be drawn.

DISCUSSION

Bone marrow shielded from *in vivo* irradiation generated more B cells *in vitro* than BM from an unirradiated mouse. This was true for 'bright' cells with large amounts of surface immunoglobulin but not for 'dull' cells with small amounts. It was best seen when BM cells taken 24 h after irradiation were kept in culture for a further 24 h. However, it still occurred when cells were taken from 6 h to 4 days after irradiation and cultured for 24 h (data not shown).

One of the problems of assessing sIg positive cell production *in vitro* is the subjective nature of ordinary fluorescent microscopy especially when attempts are made to classify BM B cells by the intensity of their staining, which is known to exhibit a marked heterogeneity (Osmond & Nossal, 1974; Yang, Miller & Osmond, 1978; Lau, Melchers, Miller & Phillips, 1979). To overcome this difficulty we used a fluorescent-activated cell sorter.

In these studies, the number of B cells found in the experimental and control cultures may be expressed either as a percentage of the total viable (or total) cell count or as an absolute number based on the Coulter counter count and corrected for dead cells by dye exclusion. In fact, the settings of the FACS excluded dead cells from the assessment of surface immunoglobulin and for this reason the viable cell count is a better reference point than the total cell count (see Materials and Methods). However, total and viable cell numbers were similar in the experimental and control groups and the conclusion that the number of bright B cells was increased in irrad-shielded mice is unaffected by the mode of calculation, i.e whether relative or absolute cell numbers are used and whether they are referred to viable or total cell counts.

The disadvantage of basing conclusions on the percentage of B cells is that this measure, unlike the absolute cell count, is influenced both by the number of B cells and the survival of non-B cells. Selective death of non-B cells may simulate an increase in B-cell numbers. Hence, absolute B-cell counts are to be preferred.

What is the significance of the absolute increase of the number of bright sIg positive cells observed in the culture of BM cells from irrad-shielded mice?

It is likely that nearly all sIg positive cells found *in vitro* were B cells and not monocytes. Very few (< 0·7%) sIg positive cells were found in monocyte-rich populations of larger cells and none of the sIg positive fluorescent cells detected by microscopy exhibited endogenous peroxidase activity characteristic of monocytes. Moreover, in normal mouse BM, 97%–98% of all antiglobulin binding cells are lymphocytes (Osmond & Nossal, 1974).

The additional bright B cells found in the culture of the experimental (irradiated) group did not arise from proliferation *in vitro*. The evidence for this is that very few of the B cells (< 2%) incorporated ^{3}H *in vitro* and hydroxyurea did not prevent the B-cell increase. It is likely that the bright B cells arose from the dull B cells. This is suggested by a comparison of the control and experimental cultures at 24 h. At that time the total number of B cells was similar in both groups. However, the number of dull cells was significantly lower

and the number of bright cells much higher in the experimental group. The simplest explanation is that the dull cells become bright cells.

Two separate processes may contribute to the regeneration of B cells following irradiation. There may be increased cell proliferation, and an increased rate of B-cell differentation or maturation. The present results provide a system for studying the second process. It is likely that increased cell proliferation also plays a role but the precise stage of B-cell development at which it occurs following irradiation is not yet known.

The nature of the stimulus for the increased appearance of B cells following irradiation is unclear. At a physiological level it might arise from a homeostatic mechanism triggered by the destruction of B cells or other cells. Alternatively, the entry of antigen from damaged mucosae may be monitored by a system which then stimulates B-cell production. At a cellular level, the problem is whether the increased appearance is due to the production of stimulatory factors or the removal of suppressor factors. The situation may be complicated, as in the case of CFU-S proliferation, which is controlled by both stimulatory and inhibitory factors (Lord *et al.*, 1978). Further experiments will be necessary to distinguish between these possibilities.

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