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IgG anti-hapten antibody secretion *in vitro* commences after extensive precursor proliferation

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Summary. We have investigated whether the intermediate cells that arise in primed mice after boosting require further cycles of division in culture before maturation into IgG antibody secreting cells. Killing of dividing cells between days 1–4 in culture, by exposure to BUdR–uv irradiation ablated the high IgG response observed on day 5 in control cultures. After T cell removal and replacement by a soluble factor (TRF) similar results were obtained. Thus B cell division over an extended period occurs prior to the appearance of IgG secreting cells. Furthermore, autoradiography of plaques from cultures briefly exposed to [^{3}H]-thymidine before harvest showed that some antibody secreting cells were synthesizing DNA at the time of assay.

INTRODUCTION

Previously, we have shown that primed spleen cells which contain a memory cell population with the ability to give high IgG responses on adoptive transfer, fail to give more than a marginal IgG response *in vitro* in a day 5 Mishell-Dutton culture (North & Askonas, 1976). To obtain high IgG/IgM antibody ratios *in vitro* it was necessary to boost the primed donors several days before killing (Henry & Trefts, 1974; North & Askonas, 1976). Here the responding cells in culture were shown to be part way along their developmental pathway from B memory to IgG antibody secreting cells. Further differentiation of this intermediate into an IgG secreting cell *in vitro* could conceivably involve either direct transformation or further cell proliferation. We therefore determined whether proliferation occurred in culture during the maturation of intermediate cells into those secreting IgG antibody. Our results indicate that even several days after *in vivo* stimulation extensive cycles of division are still necessary before IgG secretion commences.

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MATERIALS AND METHODS

Antigens, animals and priming

CBA/Ca mice, bred in the SPF colony at Mill Hill, were primed with a single intraperitoneal (i.p.) injection of 100 μg of alum precipitated Dinitrophenylated Keyhole Limpet Haemocyanin (DNP_{91}-KLH) and 2×10^9 *Bordetella pertussis* organisms 6–12 weeks before being killed. Secondary (boosting) injections of 20 μg of soluble DNP-KLH were given 7 days prior to being killed.

Tissue culture

The system of Mishell & Dutton (1967) was used with modifications as described by North & Askonas

(1976). 35 mm Petri dishes (Falcon Plastics) were used for all cultures and each contained 10^7 spleen cells in 1·0 ml of medium.

BUdR treatment

On different days as indicated (Figs 2 and 3) 3 μg of 5-bromo-2-deoxyuridine (BUdR) was added to cultures and these were further incubated for 24 h in a darkened box (Zoschke & Bach, 1972). Subsequently, cultures were exposed to ultra-violet (uv) irradiation for 30 min by being placed 60 mm below 2 Phillips 20 W/08-300-400 nm fluorescent tubes. The cells were then washed twice, resuspended in full tissue culture medium, and replaced in their original culture dishes. Control cultures given either BUdR without light exposure or uv light treatment alone were also washed as indicated above.

Depletion of T cells and their replacement by TRF

Spleen cells were depleted of T cells by incubation with Goat anti-T cell serum and rabbit complement as described by North, Kemshead & Askonas (1977). T cell replacing factor (TRF) (the supernatant of an allogeneic mixed lymphocyte reaction) was prepared according to the method of Schimpl & Wecker (1973). Details of the method of preparation are given in North *et al.*, (1977). TRF was added to cultures at 50% by volume. In all cultures that were washed free from BUdR, TRF was replaced for the subsequent culture period.

Antibody-forming cell assay

A plaque-forming cell (PFC) assay was performed on microscope slides using Trinitrophenyl (TNP)-coated horse red blood cells as described by Dresser & Greaves (1973).

Cell killing by high specific activity [^{3}H]thymidine

Cells were incubated for 24 h periods with 10 μCi of [^{3}H]thymidine (19 Ci/mM) as described by Dutton & Mishell (1967). Further [^{3}H]thymidine incorporation was blocked either by washing cultures and resuspending in medium containing 100 μg/ml unlabelled thymidine, or by the simple addition of either 30 or 100 μg/ml of unlabelled thymidine for the remaining culture period.

Autoradiography of plaque forming cells

Cells on day 5 of culture (approx. 4×10^6 cells/ml) were incubated for 4 h at 37° with either 5 or 10 μCi of [^{3}H]thymidine (18·4 Ci/mM). The cells were then harvested, washed three times and assayed for IgG PFC. The slides obtained were fixed in glutaraldehyde/saline, copiously washed and dried before coating with Ilford K5 nuclear emulsion (Rogers, 1973). Following exposure for 4 days at 4°, slides were developed using Kodak D19 developer (4 min at 22°) and fixed with a 1:3 dilution of Kodak Rapid fixer A in water. The number of radiolabelled PFC was determined at ×400 magnification using a Zeiss light microscope.

RESULTS

[^{3}H]Thymidine incorporation by spleen cell cultures

In order to determine whether proliferation occurred in culture and was dependent upon added antigen, primed and boosted spleen cells were incubated with 1 μCi of [^{3}H]thymidine (1 Ci/mM) for 24 h periods. Maximum thymidine incorporation occurred within 48 h of beginning the cultures and then rapidly declined (Fig. 1). Although the development of IgG anti-DNP secreting cells is totally dependent on the presence of antigen, no significant difference was observed in [^{3}H]thymidine incorporation between cultures either with or without antigen. Therefore, any proliferation occurring in the small

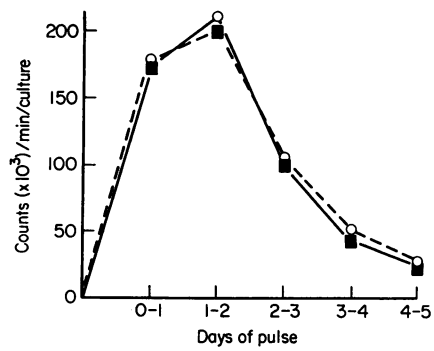

Figure 1. Incorporation of [^{3}H]thymidine by primed and boosted spleen cells. Cultures were incubated with 1 μCi of [^{3}H]thymidine (1 Ci/mM) for 24 h pulses on days indicated. The cells were then harvested, precipitated onto glass fibre discs using 10% trichloracetic acid, washed with alcohol, and ether dried. Radioactivity was determined using Zimm scintillation fluid in a Beckman Model LS200 scintillation spectrometer. (■——■) Cultures containing antigen; (○- - -○) cultures without antigen.

In this experiment on day 5, cultures containing antigen gave 42729 (s.e. 1·02) IgG plaques whereas without antigen 4571 (s.e. 1·16) IgG plaques were detected.

population of precursors maturing into IgG anti DNP secreting cells, would be masked by the high levels of antigen-independent cell division detected in culture.

Elimination of dividing cells using high specific activity [^{3}H]thymidine

During periods when precursors of PFC are synthesizing DNA *in vitro*, inclusion of high specific activity [^{3}H]thymidine into the medium will lead to their death and a consequent reduction in PFC numbers. Using the protocol established by Dutton & Mishell (1967) we confirmed the observation that IgM PFC arise in culture from dividing precursors and that sensitivity of these precursors to [^{3}H]thymidine decreased with time in culture, suggesting that some are no longer dividing by day 3–4. Addition of unlabelled thymidine (30–100 μg/ml) to cultures or the addition of [^{3}H]thymidine in the presence of excess unlabelled thymidine (30–100 μg/ml) had little effect on the IgM PFC response on day 5. However, using this technique to determine whether IgG precursors need to undergo extensive proliferation for maturation has proved unsuccessful. The developmental processes giving rise to IgG anti DNP PFC were found to be sensitive to concentrations of unlabelled thymidine that had no effect on IgM anti DNP PFC appearance. Whilst addition of 100 μg/ml of unlabelled thymidine on day 2 of culture did not effect maturation of IgM precursors even 25 μg/ml of nucleoside ablated the IgG PFC response. This, therefore, precluded the use of this technique to study division in IgG cultures as it is not possible to block [^{3}H]thymidine uptake by a sufficient concentration of unlabelled nucleoside.

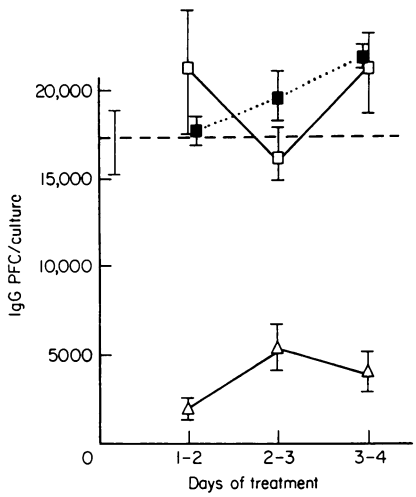

Figure 2. The requirement of cell division in culture for an IgG antibody response. IgG PFC assayed on day 5 (geometric mean of three cultures ×/÷ s.e.). (- - - - - - -) Untreated cells not disturbed throughout 5 days of culture; (△——△) 3 μg/ml BUdR for 24 h periods on days as indicated followed by 30 min uv irradiation. Cells were resuspended in fresh medium for continuation of culture; (□——□) 3 μg/ml BUdR as above but without uv irradiation; (■· · · ·■) uv irradiation without BUdR treatment.

Treatment of cultures with 5-bromo-2-deoxyuridine

Dividing cells can be selectively killed in culture following BUdR uptake and exposure to uv irradiation. Initially the concentration of BUdR not toxic to lymphocytes in the absence of photoactivation was determined. As maximal [^{3}H]-thymidine incorporation into cultures occurred between days 1 and 2 (Fig. 1), cells were incubated for this period with different concentrations of the analogue. 10 μg/ml of BUdR inhibited the appearance of IgG PFC's on day 5 of culture regardless of uv irradiation, whereas, 3 μg/ml of BUdR (24 h pulses) in the absence of uv irradiation, resulted in the same levels of IgG PFC when compared to untreated cells.

To determine whether and for how long cell division is necessary for the *in vitro* maturation of intermediate cells into those secreting IgG anti DNP antibody, cells were treated with 3 μg/ml of BUdR, uv irradiated and then recultured in the absence of the nucleoside analogue. Fig. 2 shows that selectively killing cells that incorporated BUdR during any 24 h period between days 1 and 4 of culture caused a marked diminution in the day 5 IgC PFC response. Exposure of cultures to uv irradiation only did not reduce IgG PFC on day 5, and if anything IgG production showed occasional increases. This effect was, however, inconsistent and could not be totally divorced from increases in PFCs often detected upon washing cells prior to plaque assay.

The simplest interpretation of these results is that IgG secreting cells detected on day 5 of culture arise after several proliferation cycles. However, an alternative interpretation would be that the appearance of IgG PFC depends solely on division of helper or accessory cells. Since T helper cells are

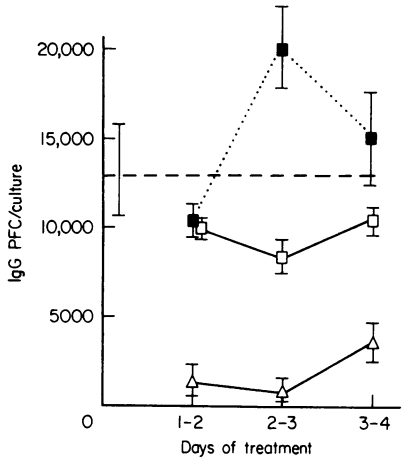

Figure 3. IgG response requirements for cell division in T cell depleted cultures. IgG PFC assayed on day 5 (geometric mean of triplicate cultures ×/÷ s.e.). (- - - - - - -) Untreated cultures of GαT treated cells given antigen and TRF; (△——△) 3 μg/ml BUdR for 24 h periods as indicated, followed by 30 min uv irradiation. Cells were resuspended in fresh medium for continuation of culture; (□——□) 3 μg/ml BUdR as above but without uv irradiation; (■· · · ·■) uv irradiation without BUdR treatment.

Control cultures containing DNP-KLH yielded 21,000 IgG PFC on day 5. After GαT treatment antigen induced 2500 IgG PFC.

essential for the development of the IgG response *in vitro* (North & Askonas, 1976) the T cell is an obvious candidate for such a role.

Proliferation in T cell depleted cultures

Addition of a supernatant from allogeneic cultures (TRF) (Schimpl & Wecker, 1973) restores the IgG response in T-cell depleted cultures (North, Kemshead & Askonas, 1977). By treating such cultures with BUdR and uv light it is possible to determine whether the observed need for cell division can be attributed to B cells. The results (Fig. 3) again show that the selective killing of dividing cells in any 24 h period between days 1 and 4 in culture leads to a greatly reduced IgG anti-TNP PFC response on day 5. In the absence of uv light treatment, cultures given 3 μg/ml of BUdR for different 24 h periods again show no significant diminution in IgG PFC when compared to untreated cells. This indicates that specific precursors of IgG secreting cells have to undergo in culture several cycles of proliferation prior to their maturation.

[^{3}H]Thymidine incorporation into IgG PFC

BUdR/uv treatment of cultures cannot resolve any requirement for proliferation just prior to the onset of antibody secretion as it is possible that cells incorporating the analogue could transiently continue to secrete antibody. To investigate proliferation requirements late in culture, day 5 cells were pulsed for 4 h with [^{3}H]thymidine, assayed for PFC and analysed by autoradiography. 15–23% of PFC were identified as being radiolabelled (cells with more than 50 grains/nucleus) and therefore appear to have synthesized DNA in the 4 h period before assay (Table 1). Cultures not incubated with [^{3}H]thymidine prior to autoradiography showed no radiolabelled PFC. The number of IgG PFC found in cultures incubated with or without isotope showed no significant differences, indicating that over a short period [^{3}H]thymidine did not inhibit antibody secretion.

Table 1. Estimate of the number of PFC radiolabelled on day 5 of culture

[^{3}H]Thymidine/culture (μCi)	IgG PFC/culture (s.e.)	% of PFC radiolabelled/slide*			
None	13875 (1·13)	0			
5	11999 (1·04)	22	23	15	
10	10406 (1·17)	21	20	19	20

Cells on day 5 of culture were incubated with [^{3}H]thymidine (18 Ci/mM) for 4 h prior to plaque assay and autoradiography. Radiolabelled plaques were estimated by light microscopy (> 50 grains/nucleus), whereby we consistently identified 65–80% of the total number of PFC.

* We estimate 89% of the PFC to be secreting IgG antibody.

DISCUSSION

Secondary responses to antigen obtained either *in vivo* or upon adoptive cell transfer result in the secretion of high levels of IgG antibody in comparison to IgM. However, low IgG/IgM ratios are obtained *in vitro* unless mice are given a boosting injection of antigen 5–7 days prior to death (North & Askonas, 1976). Here the responding cell in culture is not a true memory cell but a partially differentiated or intermediate cell type not yet capable of antibody secretion. These studies were instigated to analyse whether a direct transformation of intermediates into antibody-secreting cells occurred in culture or whether at this late stage of the IgG response, maturation was still preceded by a requirement for cell proliferation.

uv Irradiation and subsequent killing of dividing cells that had incorporated BUdR for different 24 h periods between days 1 and 4 consistently resulted in a 75–90% reduction in IgG PFC (Figs 2 and 3). Several cycles of cell division therefore appear obligatory for intermediate cells to mature into plasma cells secreting IgG antibody. We do not envisage that many IgG antibody secreting cells arise by direct transformation of precursors particularly as very few IgG anti-TNP PFC are detected over the first 3 days of culture.

Extending studies to day 5 of culture have further indicated that at least 20% of PFC are actively synthesizing DNA 4 h prior to plaque assay (Table 1). These observations suggest that either IgG PFC arise by division of other antibody-secreting cells on days 4–5, or that proliferation is still required during this period to enable maturation and the onset of IgG secretion. To account for the increases in IgG PFC often seen in experiments between days 4 and 5 we favour the latter interpretation. We cannot accurately quantify the number of proliferating cells on day 5, as during a 4 h pulse, all dividing cells may not enter their phase of DNA synthesis. Extended incubation periods with high specific activity [^{3}H]thymidine were precluded because this eventually proved lethal to dividing cells. It was found necessary to use high specific activity [^{3}H]thymidine for autoradiography of PFC as with low specific activity isotope (1 Ci/mM) grain counts were very low (<9 grains/nucleus). Because the PFC are embedded in an agarose layer we estimate that the average distance electrons have to travel from cell nucleus to photographic emulsion is of the same order as the penetrating range of the most energetic electrons emitted from [^{3}H]-thymidine (Ada, Humphrey, Askonas, McDevitt & Nossal, 1966). Electrons with such a range represent only approximately 20% of the total emitted from the isotope (Ada *et al.*, 1966) and therefore on a directional basis only a maximum of 10% of total distintegrations are capable of penetrating the photographic emulsion. The high specific activity [^{3}H]thymidine gave a low background (<3 grains/nucleus) and 95% of the labelled cells had 50 grains or more in their nucleus and thus were easily enumerated.

Attempts to kill IgG precursors by the method of Mishell & Dutton (1967) proved to be unsuccessful as these cells were found to be sensitive to levels of unlabelled thymidine that did not inhibit IgM precursors. This suggests that IgG and IgM precursors are not metabolically identical. However, our conclusion that precursors have to complete several cycles of proliferation before maturation into IgG secretory cells parallels in many respects studies undertaken on IgM cultures (Kettman, Yin & Dutton, 1973).

Hünig, Schimpl & Wecker (1974) have shown that up to 90% of IgM PFC are still dividing 6 h prior to plaque assay on day 5. Not all IgM PFC arise by this process however, as Sulitzeanu, Marbrook & Haskill (1973) have demonstrated that cells secreting IgM early in culture can develop by direct transformation from precursors. Our data obtained by killing dividing cells by either the 'hot pulse' technique of Dutton & Mishell (1967) or by BUdR-uv treatment suggests that although IgM antibody secreting cells mature through precursor division and differentiation the number of cycles in this process is not as great as for IgG precursors. Certainly, whilst primary and secondary IgM responses can be obtained *in vitro* a boosting injection has to be given to primed mice 5–7 days before being killed to obtain a high IgG response after 5 days in culture. We have suggested that the boost causes B memory cells to divide to become cells with low buoyant density capable of rapid IgG response *in vitro* (North & Askonas, 1976). Further proliferative cycles are then necessary in culture before maturation into IgG antibody secreting cells occurs. In these experiments, as part of the maturation of B memory cells to IgG antibody secreting cells occurs *in vivo*, it is not possible to determine the total number of division/differentiation cycles

involved in this process, although with an estimated average division time of 15 h, 5 cycles of division would occur in the 72 h between days 1 and 4 of culture and prior to the onset of antibody secretion. Support for the model that IgG precursors undergo more proliferation than IgM comes from mitogen stimulated cultures. IgG production can be induced in low density cultures (Kearney & Lawton, 1975) after extensive cell proliferation (Askonas & North, 1976) as well as in high density cultures subsequent to several phases of proliferation over 2–3 weeks (Zauderer & Askonas, 1976).

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