

## AUTORADIOGRAPHIC STUDIES ON THE PROLIFERATION OF ANTIBODY-PRODUCING CELLS IN VITRO\*

By T. HÜNIG, A. SCHIMPL, AND E. WECKER

(From the Institute of Virology, Würzburg, West Germany)

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In the primary in vitro immune system described by Mishell and Dutton (1), convincing evidence was provided that proliferation of precursor cells precedes the appearance of hemolysin excreting, i.e., plaque-forming cells (PFCs).<sup>1</sup> As shown by the "hot pulse" technique (2) the expansion of antigen-reactive clone cells appears to be a normal and necessary event in the humoral immune response in vitro, just as in vivo (3).

An optimal in vitro immune response to sheep red blood cells (SRBC) only occurs in the presence of T cells (4). We wanted to study whether or not the helper function is essential for the proliferation of antigen-reactive B precursors without disturbing a normal immune response by hot pulses or inhibitors. For this purpose it was necessary to develop an assay based on single-cell analyses rather than on values describing the whole population. Here we describe an autoradiographic technique which allows for direct study of the proliferation of PFC precursors throughout the culture period in the presence or absence of helper function.

### Materials and Methods

*Mice.*—The strains of mice used were, nu/nu Balb/c (Bomholtgard, Rye, Denmark), DBA/2J (Jackson Laboratories, Bar Harbor, Main), and CBA/J (Jackson Laboratories). Mice of both sexes were used. Spleen cell cultures were prepared as described by Mishell and Dutton (1) and cultured in Eagle's minimal essential medium (MEM) for normal cultures (Microbiological Associates, Inc., Bethesda, Md., Cat. no. 12125).  $1 \times 10^7$  cells in 1 ml of culture medium supplemented as given in (1) and containing 5% fetal calf serum (Flow Laboratories, Bonn, West Germany) were seeded into 30-mm plastic petri dishes and stimulated with SRBC ( $5 \times 10^6$ /dish). In cases of T-cell replacing factor (TRF) addition, 0.5 ml of the medium were carefully removed and replaced by 0.5 ml of TRF. TRF was prepared as described previously (5) and stored at  $-70^\circ\text{C}$  until use.

Pulse conditions were as described in the text or in the legends to the figures. Chase was performed by carefully removing the radioactive medium, rinsing each dish with 1 ml of non-radioactive medium, and then adding 0.5 ml of fresh medium/dish. Cells were recovered from both supernates by centrifugation at 170 *g* for 10 min. 0.5 ml fresh medium containing the sedimented cells from the washing fluids and 100  $\mu\text{g}$  cold thymidine were then added to each respective dish.

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<sup>1</sup> Abbreviations used in this paper: BSS, balanced salt solution; PFCs, plaque-forming cells; SRBC, sheep red blood cells; TRF, T-cell replacing factor.

At the times indicated, cells were harvested, washed three times in 10 ml balanced salt solution (BSS), and resuspended in the appropriate dilutions of BSS for the Jerne assay modified by Mishell and Dutton (1, 6). The number of recovered nucleated cells was determined in a Coulter Counter (Model F<sub>N</sub>, Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) using Zaponin to lyse red cells. The slides were fixed immediately after the development of the plaques by a 10-min treatment with 0.5% glutaraldehyde in phosphate-buffered saline, washed three times for 5 min in distilled water, and air-dried.

"Thick-layer" autoradiography was performed as described by B. Schultze, University of Würzburg (personal communication). Ilford nuclear emulsion K2 (Ilford Ltd., Ilford, England) was melted for 30 min at 42°C and transferred into a glass vessel kept at 30°C. After 10 min the slides were covered with the emulsion by dipping them once for 2-3 s. After an exposure of 8 or 9 days developing was carried out by bathing the slides for 20 min at 12°C in a special developer: 3.4 g Amidol (Merck, A. G. Darmstadt, West Germany), 13.5 g Na<sub>2</sub>SO<sub>3</sub>, 26.3 g boric acid, and 6 ml of 10% KBr solution made up to 1.5 l in distilled water. The autoradiographs were fixed in a solution of 30% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> for 50 min at room temperature. After rinsing, the preparations were air-dried, Giemsa-stained, and grains were counted at a magnification of 1:1,250 (Leitz Ortholux, E. Leitz, KG, Wetzlar, W. Germany). All numbers above 300 grains/cell, as well as "uncountably high" values which cannot be exactly evaluated, were arbitrarily reduced to 300 grains/cell. Therefore, values giving average numbers of grains/cell never surpass the 300-grain limit, and with heavily labeled cells underestimate the true average numbers. We generally checked around 100 PFCs and more than 200 non-PFCs/experimental group for their label.

#### RESULTS AND DISCUSSION

The autoradiography of plaque center cells in agarose poses specific problems. Depending on the isotope and the thickness of the agarose layer, a considerable portion of the emitted electrons will be absorbed and lost. Compensating for this loss by increasing either the specific or the absolute radioactivities of the thymidine, however, causes radiation damage in the incorporating cells.

Table I compares the labeling patterns obtained in antigen-stimulated spleen-cell cultures using [<sup>3</sup>H]thymidine and [<sup>14</sup>C]thymidine as DNA precursors. <sup>14</sup>C possessing the higher energy of emission is the superior isotope for the autoradiography of plaque-center cells. Under otherwise identical conditions, 1 μCi of [<sup>14</sup>C]thymidine (specific activity 495 mCi/mmol) gave a far better labeling of PFCs than 10 μCi of [<sup>3</sup>H]thymidine (specific activity 2 Ci/mmol). Also, the number of PFCs was less reduced by 1 μCi of [<sup>14</sup>C]thymidine than by 10 μCi of [<sup>3</sup>H]thymidine. As subsequent experiments showed, 1 μCi [<sup>14</sup>C]thymidine of the specific activity 53 mCi/mmol still labeled the cells very satisfactorily while causing virtually no suicide. In all subsequent experiments we used these conditions for labeling the cells but still included a suicide control in each experiment.

The plaque center cells arising from precursors which are triggered by antigen should preferentially incorporate the radioactive thymidine because of preferential proliferation. This is indeed the case (Fig. 1 a). [<sup>14</sup>C]thymidine added at h 72 to SRBC-stimulated DBA spleen cultures already labeled more than 90% of all the PFCs if the plaque assay was performed 6 h later. In contrast, only

TABLE I  
*The Choice of Isotopes*

Exp. no.	Radiolabeled thymidine/culture	PFC/10 <sup>6</sup> *	Label in PFCs‡
1	10 $\mu$ Ci <sup>3</sup> H	62	+
	5 $\mu$ Ci <sup>3</sup> H	77	+
	1 $\mu$ Ci <sup>3</sup> H	690	(+)§
	1 $\mu$ Ci <sup>14</sup> C	450	++
	0.5 $\mu$ Ci <sup>14</sup> C	650	+
	0.25 $\mu$ Ci <sup>14</sup> C	870	(+)
	—	2,400	—
2	1 $\mu$ Ci <sup>14</sup> C	530	++
	0.5 $\mu$ Ci <sup>14</sup> C	590	+
	—	770	—
3	1 $\mu$ C <sup>14</sup> C	680	(+)
	—	720	—

Exp. 1: Pulse, 48–120 h; plaque assay, 120 h of culture; [<sup>3</sup>H]thymidine, 2 Ci/mmol; [<sup>14</sup>C]thymidine, 495 mCi/mmol. Exp. 2: Pulse, 48–96 h; plaque assay, 96 h; [<sup>14</sup>C]thymidine, 53 mCi/mmol. Exp. 3: Pulse, 32–44 h; plaque assay 120 h; [<sup>14</sup>C]thymidine, 53 mCi/mmol. Culture conditions as in Materials and Methods, using mice of different strains.

\* PFC/10<sup>6</sup> nucleated cells.

‡ ++, more than 300 grains/PFC and +, all PFC labeled.

§ (+), labeled as well as unlabeled PFC.

about 10% of all lymphocytes not participating in plaque formation incorporate radioactivity. This result may be taken as a specificity control of the technique indicating that repair mechanisms, labeling of cells by [<sup>14</sup>C]thymidine adsorbed onto membranes, or only present in intracellular pools etc. do not significantly falsify the results.

Fig. 1 *b* shows the average number of grains found in plaque center cells whose progenitors had been exposed to [<sup>14</sup>C]thymidine for various times. After 9 h the number of grains already had reached such high values that additional incorporation during the subsequent 15 h in the presence of the label (upper curve) could not be evaluated quantitatively (see Materials and Methods). If, however, a pulse of 12 or 24 h was followed by a chase period until the time of plaque assay the number of grains/PFC was higher, the shorter the chase period. Both these findings suggest that most of the cells continued to divide up to the time of assay. If the pulse was followed by a chase period the incorporated radioactivity was diluted by subsequent divisions (lower curve). The dilution effect would, of course, become stronger with increasing time, i.e., with increasing cycles of division.

We conclude from these experiments that *in vitro* all of the PFCs originate from actively dividing precursors. This is in keeping with the results of Dutton and Mishell (2). Nossal and Mäkelä (3) had previously found that in a secondary *in vivo* response most antibody-forming cells are the results of antigen-induced mitoses; similar results were obtained by Russel and Diener (7).

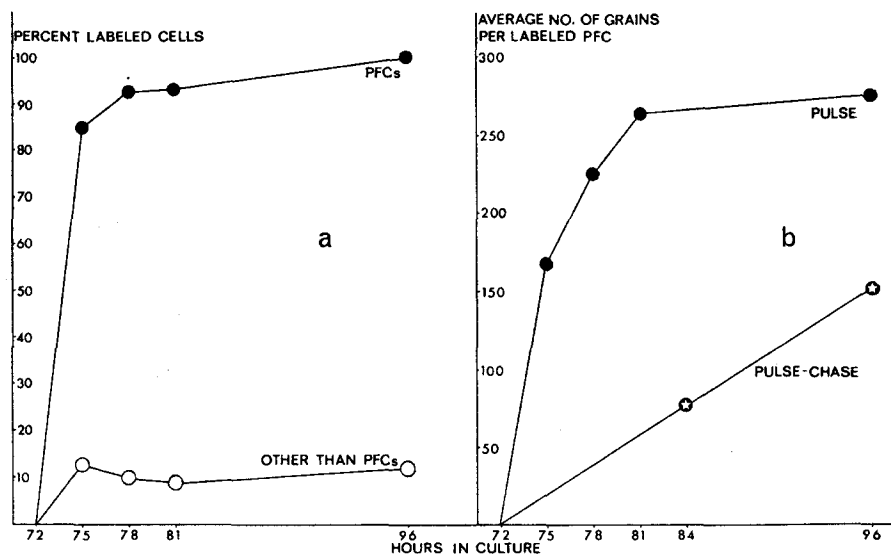


Fig. 1. Specific incorporation of  $^{14}\text{C}$ thymidine into PFCs and the effect of different chase periods. DBA/2J spleen cell cultures received  $1\ \mu\text{Ci}$   $^{14}\text{C}$ thymidine at 72 h of culture. (a) Hemolytic plaque assay carried out at 75, 78, and 81 h, label evaluated for plaque-forming (closed circles) and nonplaque-forming cells (open circles). (b) Average number of grains/PFC, closed circles show the same cells as in Fig. 1 a. To the cells represented by circled stars,  $^{14}\text{C}$ thymidine was also added at 72 h, followed by a chase at 84 h or 96 h. The plaque assay was performed at 120 h.

In contrast, Sulitzeanu et al. (8), also by applying autoradiography, found that far from all PFCs previously exposed to  $^3\text{H}$ thymidine produced visible grains. Having compared the effectiveness of  $^3\text{H}$  and  $^{14}\text{C}$  in the autoradiography of PFCs, we think that "unlabeled" PFCs in the experiments of Sulitzeanu et al. had in fact incorporated label which could not be detected.

Mäkelä and Nossal also observed that some cells which already started to produce specific antibodies still incorporated label (9). Our results suggest that in our system this may indeed be the case for the vast majority of all PFCs.  $^{14}\text{C}$ thymidine pulses given 6 h before plaque assay labeled about 90% of all the PFCs (Fig. 1 a). After a 24-h pulse practically all PFCs gave positive autoradiographs. In contrast, only about 15% of the cells not participating in plaque formation incorporated radioactivity under these conditions.

The reliability and applicability of this autoradiographic technique having been established, it was now utilized to answer a more specific question. SRBC have been shown both in vivo (10) and in vitro (4) to be T-cell-dependent antigens. The presence of T cells can be completely substituted for by a soluble TRF in the primary and secondary plaque responses to heterologous red blood cells in vitro (5). The highest number of PFCs is obtained when TRF is given only 1-2 days after commencement of B-cell cultures containing heterologous blood cell antigens from the beginning (5). Without TRF only a low background

of plaques is observed. It was therefore postulated that the T-cell helper function mediated by TRF was in fact not required for the initial triggering events of precursor cells but rather for a later step resulting in actual antibody production (11).

Consequently, proliferation of PFC precursors should occur already by contact with SRBC antigens and before T-cell helper function. This prediction was now studied by applying the autoradiographic technique described above to B-cell cultures later restored by TRF.

Spleen cultures from athymic nu/nu mice containing SRBC from the beginning were exposed to [ $^{14}\text{C}$ ]thymidine for 12-h periods (pulses) at various times. Each pulse was terminated by a chase lasting to the time of plaque assay on day 5. In all cases TRF was added 60 h after the commencement of the culture. Thus, in three samples the label was only available before, and in another sample after the addition of TRF. The results of this experiment are shown in Fig. 2. Almost 70% of the PFCs were still labeled at the end of the culture period when radioactivity was available between h 24 and 36, i.e., well before TRF was added and before any significant number of PFCs was detectable in the culture. This value increased steadily with later pulses and it reached its maximum of 95% labeled PFCs with a pulse given between h 62 and 74. This

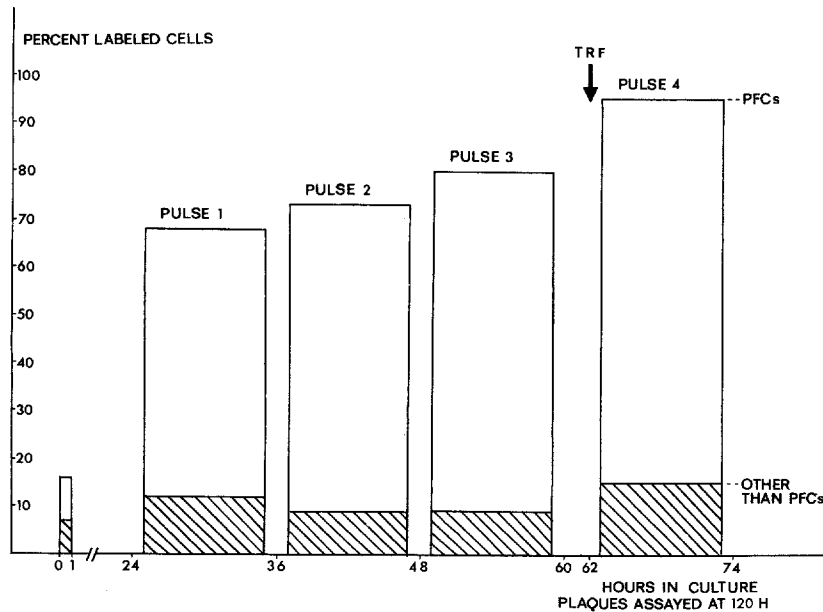


FIG. 2. Incorporation of [ $^{14}\text{C}$ ]thymidine into nu/nu spleen cell cultures. Percentage of labeled PFC (total bars) and non-PFC (shaded part) of nu/nu spleen cell cultures, pulsed before (pulses 1-3) or after (pulse 4) addition of TRF. Chase control: pulse from 0-1 h  $1 \mu\text{Ci } ^{14}\text{C}/\text{ml}$ .

increase in labeled cells is most likely due to the fact that the cells pulsed closer to the time of assay had less time to dilute their label by subsequent cell division.

In contrast, depending on the time of pulse, only 9–15% of the lymphocytes not participating in plaque formation were labeled. Unless SRBC only reacted with cells which divide anyhow, these observations suggest: (a) cells which later on produce specific antibodies to SRBC are preferentially driven into proliferation by their specific antigen and they react to it in the seeming absence of any T-cell help. (b) The helper function of TRF does not reflect itself in a significant and additional proliferogenic activation of subsequent PFCs, and clone expansion seems to proceed quite steadily during the culture period.

The effect of TRF on the numbers of PFCs obtained in B-cell cultures is striking compared to the seeming capability of PFC precursors to proliferate without T cells or TRF. It seems that in the response to heterologous blood cells *in vitro*, the main function of TRF is to help antigen-triggered dividing precursor cells to turn into antibody-producing cells. This notion corroborates pertinent results described elsewhere (11–13) and adds weight to the model suggested in these publications.

#### SUMMARY

A rapid and reliable autoradiographic technique for plaque-forming cells (PFCs) using  $^{14}\text{C}$  rather than tritiated thymidine is described. Its application to PFCs developing *in vitro* shows that (a) practically all PFCs derive from precursors dividing steadily during the culture period, (b) PFC precursors divide in the absence of T-cell helper function, and (c) at least some PFCs may continue to divide.

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