Biochemical Pharmacology, Vol. 21, pp. 59–68. Pergamon Press, 1972. Printed in Great Britain

EFFECT OF ANTIBIOTICS ON THE DEVELOPMENT OF ANTIBODY-PRODUCING CELLS, *IN VITRO**

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(*Received* 19 *February* 1971; *accepted* 4 *June* 1971)

Abstract—Using the Mishell–Dutton technique† for culturing mouse spleen cells to produce hemolysin plaque-forming cells (PFC) *in vitro*, actinomycin D, puromycin or cycloheximide were added daily to determine antibiotic-sensitive sites. The culture period was functionally divided into three temporal phases: (a) An initial phase (0–24 hr) of cultivation was most sensitive to antibiotic action (b) An intermediate phase (24–72 hr) of cultivation, whose biological function is largely undetermined, was sensitive to low concentrations of antibiotics (c) A third or functional phase (72–96 hr) of cultivation was least sensitive to antibiotic action. Our data are consistent with the notion that early developmental cell types are most sensitive to antibiotic action and, as these mature into functional cells, are less affected by the antibiotics.

ANTIBIOTIC effects on antibody-formation in intact animals were found to vary.[1–5] Actinomycin D partially inhibited the primary response to sheep red blood cells (SRBC) in mice; it delayed the appearance of antibody in the serum of rats undergoing a primary response, but had no apparent effect on total circulating antibody production.[3] Geller and Speirs[4] showed that actinomycin D did not diminish anti-toxin production to anamnestic response.

Since the interpretation of results in intact animals is hindered by the possible interchange of cells from one lymphoid organ to another, we used an *in vitro* culture technique to eliminate this possibility. Previously we reported[6] that low concentrations of actinomycin D, puromycin and cycloheximide inhibited the production *in vitro* of antibody-forming cells. Using the Mishell–Dutton technique[7] for culturing antibody-forming cells, antibiotics at 10^{-6} M or lower inhibited production of hemolysin plaque-forming cells (PFC) when the drugs were added 48 hr after cultures were initiated. However, because drugs were added at one time interval, this study excluded drug effects at other time intervals of the cultivation period. Other investigators have studied the effect of antibiotics *in vitro* on lymphoid cells from immunized animals.[8–12]

To define further the loci of inhibition with respect to the developmental phases of immunocompetent cells, the present study examined the effect of antibiotics at various temporal intervals of cell cultivation. The simple design of the experiments was to determine whether concentrations of actinomycin D, cycloheximide and puromycin, which markedly inhibited PFC *in vitro* on their addition to cultures on the second day, would demonstrate differential effects when added at other days of the culture period.

* Supported by A.E.C. C00-1632-27 and GM 15956.

† R. I. Mishell and R. W. Dutton, *J. exp. Med.* **126**, 423 (1967).

We expected that the production of antibody-forming cells was most sensitive at early time periods and, as these cells matured into functional cells, would be less affected by the antibiotics.

EXPERIMENTAL

Animals. Male C57L × A/He (LAF_1) mice between 8 and 16 weeks of age were obtained from Jackson Memorial Laboratories, Bar Harbor, Me.

Antibiotics. Puromycin dihydrochloride was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Cycloheximide (Actidione) was a gift from Upjohn Co., Kalamazoo, Mich., and actinomycin D was supplied by Merck, Sharp & Dohme, West Point, Pa.

Tissue culture media. Fetal calf serum was obtained from Reheis Chemical Co., Kankakee, Ill., and sheep red blood cells were obtained from Colorado Serum Co., Denver, Colo. Concentrates of minimum essential Eagle's medium (MEM), Hanks' balanced salt solution (HBSS), essential amino acids, non-essential amino acids, sodium pyruvate and guinea pig complement were obtained from Grand Island Biological Co., Grand Island, N.Y.

Preparation of short-term culture of PFC. Cell culture conditions for production of PFC, *in vitro*, were described by Mishell and Dutton.[7] Details of the procedure used to assay hemolysin plaques were reported in an earlier study.[13] As a routine procedure, $1{\cdot}5 \times 10^7$ dispersed cells teased from mouse spleens were planted with sheep red blood cells in 1·0 ml of Eagle's medium. Antibiotics in 50-μl quantities were added to cultures on consecutive days and the cultures were assessed for PFC on the fourth day of culture. Falcon tissue culture plastic ware was used in this study.

Drug-pulsing of M (adherent) and L (non-adherent) cell fractions. For separation of L and M cells, we used Mosier's[14] technique. Briefly, cells adherent (M) to plastic tissue culture dishes were separated from cells that did not adhere (L). Drugs were added to L and M fractions and incubated for a period of 40 min. Excess drug was removed in the following manner: for M cells adhering to tissue culture dishes, the supernatant fluid containing drug was removed by suction, washed two times with 1·0 ml of HBSS, swirled, centrifuged and removed again by suction. L cells in plastic test tubes were centrifuged at 800 rev/min in a PR-2 International refrigerated centrifuge. After removing the supernatant solution by suction, cells were washed two times with 2·0 ml of HBSS containing 10% fetal calf serum. The drug-treated cell fractions were then combined with their untreated partners for a cultivation of 4 days.

Drug pulsing. Drugs were pulsed at daily intervals in the following manner: after 40 min of contact with cells in 35 mm tissue culture dishes, the supernatant fluid was removed with a Pasteur pipette and tissue culture medium was added to cultures.

Incorporation of radioactive RNA precursors into RNA (RNA synthesis). Two μc of a solution containing tritium-labeled guanosine, cytidine, adenosine and uridine were added to L and M spleen cell cultures. The tritium-labeled compounds were purchased from Schwarz Bioresearch, Inc., Orangeburg, N.Y. After the incubation period, M cells were scraped from tissue culture dishes with a plastic policeman and placed in test tubes. The test tubes containing L and M cells were then centrifuged at 1000 rev/min in a refrigerated centrifuge for 5 min. After one wash in HBSS and a subsequent centrifugation, one-fifth of the cells (contained in 50 μl of HBSS) were

placed on 24 mm filter paper disks and processed for RNA synthesis by Bollum's technique.[15] Tritium-labeled RNA was counted on a Packard scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

RESULTS

Actinomycin D. The effects of adding actinomycin D on consecutive days are shown in Fig. 1. Figure 1a reveals that, at a concentration of 4×10^{-8} M, actinomycin D profoundly inhibited the formation of PFC when added on days 0, 1 and 2 of the culture period. A 24-hr contact with actinomycin D (i.e. added on day 3) resulted in a 50 per cent inhibition of PFC production. Actinomycin D, in concentrations which were one-fifth of that used above (i.e. $8{\cdot}0 \times 10^{-9}$ M, Fig. 1b), showed a pattern of response similar to Fig. 1a although the degree of inhibition was less with 24- and 48-hr contact with the antibiotic.

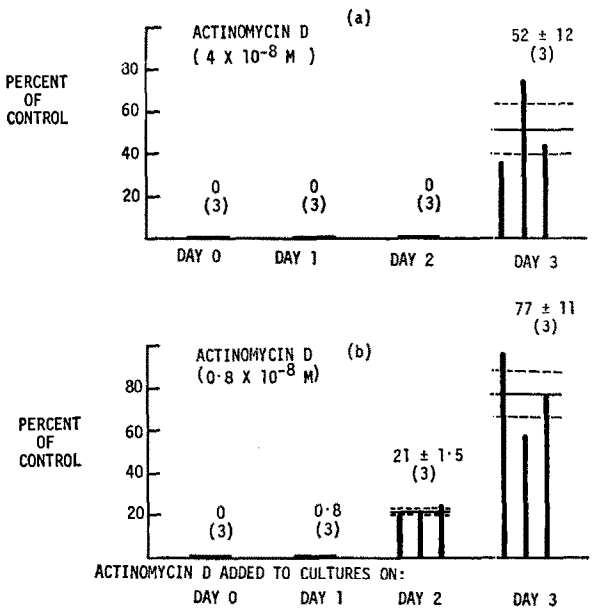

FIG. 1. Effect of adding actinomycin D on days 0, 1, 2 and 3 on the production of PFC. Each vertical line represents one experiment. Horizontal lines and numbers represent mean ± standard error of the mean. Number of experiments for each day is in parentheses.

Puromycin. Figure 2 shows the effects of adding puromycin on successive days to cultured cells. A puromycin concentration of 5×10^{-6} M was markedly inhibitory to production of PFC when the antibiotic was added on the first 3 days of culture, whereas the addition of puromycin on day 3 resulted in PFC production which was 24 per cent of control values. At lower concentrations, i.e. $2{\cdot}5 \times 10^{-6}$ M (Fig. 2b) and 1×10^{-6} M (Fig. 2c), puromycin was markedly effective in suppressing formation of antibody-producing cells when they were in contact with cells from the first day of culture (Fig. 2c). Whereas the concentration of $2{\cdot}5 \times 10^{-6}$ M was effective (viz. 3·6 per cent of control) when added on day 2, a concentration of 1×10^{-6} M was not effective (84 per cent of control). As seen in Figs. 2a, b and c, puromycin-cell contact for the last 24-hr period was the least effective of any time interval.

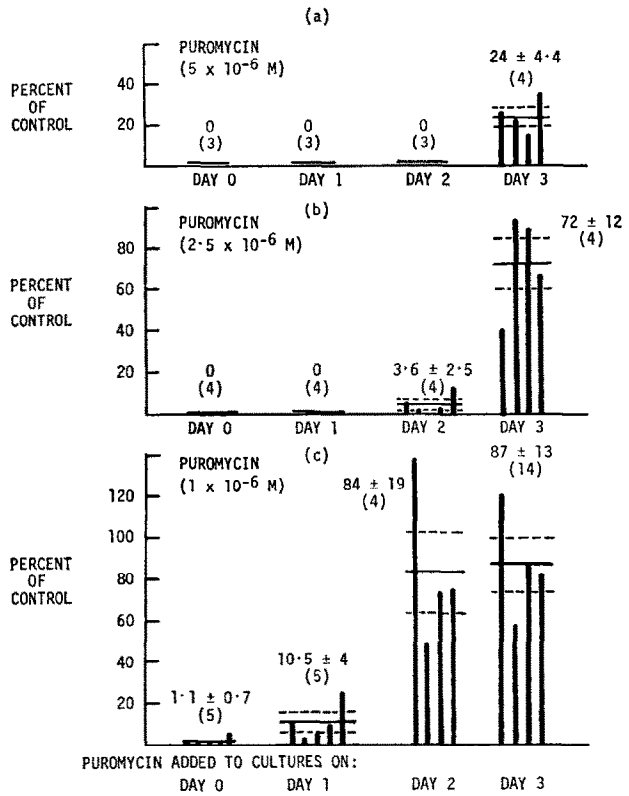

FIG. 2. Effect of adding puromycin on days 0, 1, 2 and 3 on the production of PFC. Each vertical line represents one experiment. Horizontal lines and numbers represent mean ± standard error of the mean. Number of experiments for each day is in parentheses.

Cycloheximide. Results of studies on the addition of cycloheximide on successive days of culture are shown in Fig. 3. At $3{\cdot}6 \times 10^{-8}$ M, cycloheximide was effective in suppressing production of antibody-producing cells when the drug was added on days 0 and 1. At the same concentration, cycloheximide added on days 2 and 3 was 26 and 41 per cent of controls respectively. It should be noted that, in general, cycloheximide was not as inhibitory to PFC production as actinomycin or puromycin when the drug was added on days 1 and 2.

Pulsing of antibiotics. A difficulty in our experiments was that once they were added, drugs remained in contact with cells during the remainder of the culture period. To overcome this difficulty, drugs were pulsed at daily intervals. Results are shown in Table 1; the general pattern of inhibition was similar to that observed when drugs remained in the media throughout the culture period. One notable difference was that higher concentrations of antibiotics were needed to exert their inhibitory action; in particular, much higher concentrations of cycloheximide were employed to exert the same degree of inhibition as when cycloheximide was left in contact with cells after its addition.

Antibiotics on L and M cells. Studies by Mosier[14] and Pierce and Benacerraf[16] showed that at least two functionally different cell populations, L (non-adherent) and

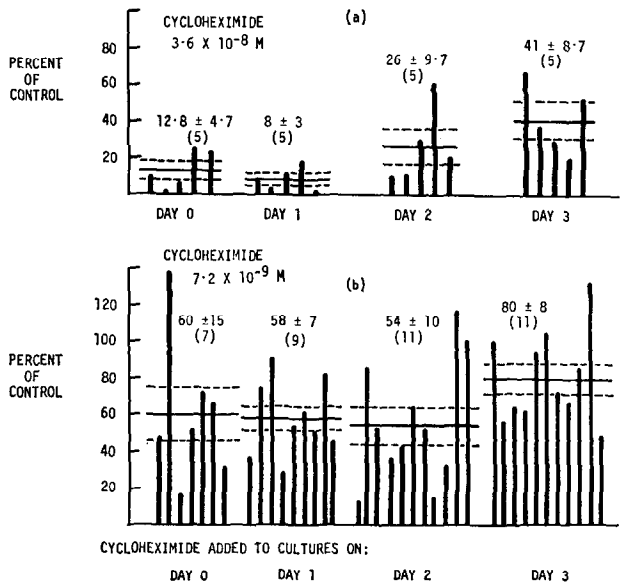

FIG. 3. Effect of adding cycloheximide on days 0, 1, 2 and 3 on the production of PFC. Each vertical line represents one experiment. Horizontal lines and numbers represent mean ± standard error of the mean. Number of experiments for each day is in parentheses.

M (adherent), are required for PFC production, *in vitro*. Because of their ability to engulf foreign particulates (greater than 90 per cent of the M fraction), M cells were designated as macrophages. This separation enabled us to determine if one cell population was more sensitive to antibiotic action than the other. Antibiotics were pulsed with drugs for 40 min as outlined in the Experimental section.

As shown in Table 2, a 40-min pulse of actinomycin D was markedly effective to both L and M fractions. The additive effect of normal L plus normal M cells was 32 per cent of control (26 + 6 = 32). Whether L cells or whether M cells were pretreated with actinomycin D and cultivated for 4 days with their non-treated partners (groups 4 and 5), the PFC were reduced to 0–2 per cent of control. Cycloheximide pretreatment indicated some differences; when L cells were pretreated (group 5) the activity was diminished to 46 per cent of control compared to 23 per cent (group 6) when M cells were pretreated with cycloheximide. Results of pulsing L and M cells separately with puromycin indicated that the degree of inhibition was not significantly different (40 and 53 per cent of control for groups 8 and 9 respectively).

Antibiotics on RNA synthesis of L and M cells. The preceding experiments established that the initial phase of antibody-formation was highly susceptible to the inhibitory action of the antibiotics. We, therefore, studied the effects of antibiotics on RNA synthesis of the initiating cells. Two drug–cell contact times were employed; schemes A and B were used to test the effect of antibiotics on RNA synthesis of L and M cells. Scheme A was a 1-hr preincubation of cells with drugs before a 4-hr pulse with tritium-labeled RNA precursors. Scheme B was an 18-hr preincubation of cells with drugs before a 4-hr pulse with tritium-labeled RNA precursors.

Experimental evidence shown in Table 3 indicates that: (a) with longer drug–cell

TABLE 1. EFFECTS OF A 40-min PULSE OF ANTIBIOTICS ON THE PRODUCTION OF PFC

Drug pulsed on	Actinomycin D (0·02 μg/ml; 0·016 μM)*		Puromycin (1·5 μg/ml; 0·8 μM)†		Cycloheximide (0·025 μg/ml; 0·087 μM)*	
	PFC per dish	Percentage of control	PFC per dish	Percentage of control	PFC per dish	Percentage of control
Day 0	80	70	0	0	685	66
Day 1	795	69	0	0	440	44
Day 2	1380	120	220	10	720	69
Day 3	690	60	920	43	570	55
Not pulsed (control)	1150	100	2150	100	1040	100
	Actinomycin D (0·05 μg/ml; 0·04 μM)*		Puromycin (7·5 μg/ml; 4·0 μM)†		Cycloheximide (15 μg/ml; 51·0 μM)*	
	PFC per dish	Percentage of control	PFC per dish	Percentage of control	PFC per dish	Percentage of control
Day 0	0	0	0	0	0	0
Day 1	0	0	0	0	35	3
Day 2	125	12	0	0	1030	89
Day 3	720	69	20	1	710	62
Not pulsed (control)	1040	100	2150	100	1150	100

* Average of three experiments.
† Average of five experiments.

TABLE 2. EFFECT OF A 40-min ANTIBIOTIC PULSE OF L OR M SPLEEN CELLS ON THE PRODUCTION OF PFC

Group	PFC per dish*	Percentage of control*
1. L plus M	1170	100
2. L alone	304	26
3. M alone	70	6
4. AD-treated-L plus M	0	0
5. L plus AD-treated-M	20	2
6. CHX-treated-L plus M	540	46
7. L plus CHX-treated-M	270	23
8. PUR-treated-L plus M	470	40
9. L plus PUR-treated-M	620	53

* Represents average values obtained from five individual experiments in which three spleens were pooled for each experiment. Drug concentrations: actinomycin D (AD)–0·05 μg/ml; cycloheximide (CHX)–1·5 μg/ml; puromycin (PUR)–1·5 μg/ml.

TABLE 3. EFFECT OF ANTIBIOTICS ON RNA SYNTHESIS OF L AND M CELLS OF MOUSE SPLEEN*

	Drug	Concn (μg/ml)	Scheme A			Scheme B		
			Experiment no.		Percentage of control (average of two experiments)	Experiment no.		Percentage of control (average of two experiments)
			(counts/min/disk)†	(counts/min/disk)†		(counts/min/disk)†	(counts/min/disk)†	
L Cells	Control		3746	930		1920	3815	
	Actinomycin D	0·02	3135	999	95	1205	2900	69
		0·05	3365	980	97	929	2118	52
	Cycloheximide	1·5	2268	999	84	1270	2283	62
		15·0	1868	312	41	406	597	19
	Puromycin	1·5	2155	619	61	378	874	22
		15·0	1014	218	25	115	95	4
M Cells	Control		1340	340		2550	4440	
	Actinomycin D	0·02	440	273	58	724	916	25
		0·05	605	269	63	523	740	18
	Cycloheximide	1·5	257	168	35	418	441	13
		15·0	177	63	16	182	194	5
	Puromycin	1·5	267	129	30	162	267	6
		15·0	121	101	20	65	71	1

* Two μc of tritiated RNA precursors were added to control and drug-treated cells. The sp. act. of the compounds were the following: 8-^{3}H adenosine (26 c/mole), 8-^{3}H guanosine (4·7 c/mole), 5-^{3}H cytidine (6·0 c/mole) and 5-^{3}H uridine (4·0 c/mole). Scheme A represents 1-hr preincubation with drug before a 4-hr pulse with tritiated RNA precursors. Scheme B represents 18-hr preincubation with drug before a 4-hr pulse with tritiated RNA precursors.

† Represents counts per minute per aliquot (amounting to one-fifth of incubated cells) placed on filter paper disks.

contact time, RNA synthesis is inhibited to a greater extent; (b) RNA synthesis in M cells was inhibited to a greater extent as compared with L cells when the same concentrations of antibiotics were used.

Actinomycin D. No significant differences in the inhibition of RNA synthesis were observed in either L or M cells with the two concentrations of actinomycin D employed. In both L and M cells, longer contact with the drug resulted in a greater inhibition of RNA synthesis (compare 58–63 vs. 18–25 per cent of control for M cells and 95–97 vs. 52–69 per cent of control for L cells).

Cycloheximide. Using the antibiotic, higher concentrations of the antibiotic inhibited RNA synthesis to a greater extent than the lower concentration. Also, the longer duration of drug-contact (scheme B) tended to diminish RNA synthesis more than a shorter contact (scheme A).

Puromycin. The pattern of inhibition of puromycin was similar to cycloheximide; M cells were inhibited more than L cells, higher concentrations were more inhibitory than lower concentrations, and longer duration of drug contact resulted in a higher degree of inhibition.

DISCUSSION

An important aspect of the present study was to study the sequential blockade of the developmental phases of antibody production. We assumed the validity of the molecular mechanisms of action of the three antibiotics on macromolecular synthesis, viz. actinomycin D on DNA-dependent RNA synthesis,[17,18] puromycin[19–21] and cycloheximide[22] on protein synthesis. Because developmental phases of antibody production involve the synthesis of DNA, RNA, and proteins at several temporal intervals, antibiotics were used as biochemical probes to further dissect the complex immune response. Darnell[23] similarly has used antibiotics as biochemical probes in his studies on ribosome biogenesis of HeLa cells.

We, as well as others,[7,16,24] found the peak of PFC production occurred 4 days after planting. Nossal *et al.*[25] recently showed that mouse peritoneal cells produced hemolytic plaques within hours of explantation; however, normal spleen cells did not form plaques in their culture technique. Based on our experiences with the Mishell and Dutton technique,[7] the culture period can be functionally, if arbitrarily, divided into three temporal periods. We recognize that these phases cannot be clearly demarcated; rather, they represent a continuum between temporal phases. Developmental cell types may exist in more than one temporal phase.

Antibiotics were most effective on the first or initiating phase of PFC production (0–24 hr of culture). Thus, daily additions (Figs. 1, 2 and 3) or pulsing (Table 1) of the three antibiotics on day 0 showed the most inhibition; the same concentrations of drugs became less effective when they were added on succeeding days of culture. Cell proliferation is ascribed to this period because agents which inhibit "cycling cells"* are effective in suppressing PFC when they are added at these intervals. In this initiating phase, a number of studies[14,16,17] showed that at least two functionally different cell types in mouse spleen are required to initiate PFC production. Roseman[26] demonstrated that adherent cells (M) were resistant to radiation and that non-adherent cells

* "Cycling cells" denote cells that are in the DNA synthetic cycle, viz. G_1, S, G_2 and M.

(L) were extremely sensitive to radiation. It is noteworthy that the antibiotics, particularly actinomycin D, inhibited the contribution of L and M cells to produce PFC in the reconstituted L and M experiments (Table 2). Collectively, the findings of Roseman and our data suggest that whereas cell proliferation of M cells is not a requisite contribution, a DNA-dependent RNA synthesis is necessary for the "immune function" of M cells in the reconstituted L and M experiments. Further, our studies demonstrated that low concentrations of actinomycin D inhibited RNA synthesis of M cells (Table 3).

To inhibit both the production of PFC in the reconstituted L and M experiment (Table 2) and RNA synthesis of L and M cells (Table 3), higher concentrations of cycloheximide or puromycin are needed; for these reasons we presently feel that the inhibition of DNA-dependent RNA synthesis plays a more prominent role in inhibiting the immune function of M cells. Since low doses of radiation inhibited L cell contribution to the reconstituted L and M experiments,[26] antibiotic inhibition of the immune function of L cells cannot be dissociated from their ability to inhibit cycling cells.

The second or intermediate phase was arbitrarily demarcated between the twenty-fourth and seventy-second hour of cultivation. Since the biological functions are largely undetermined, this phase was assigned one-half of the cultivation period. There is some experimental support for the limits assigned to this phase. Pierce and Benacerraf[16] showed that the immune function of M cells is no longer needed after the first day of culture. At the other end, PFC begin to appear on the third day but reach peak levels on the fourth day. Cell proliferation is also ascribed to this period since agents which inhibit cycling cells are usually effective in suppressing production of PFC.[6,27]

The antibiotic-resistant phase is clearly the third or functional phase, which is characterized by its mature or end-cell function (release of antibody in our case). Our collective data on daily additions and pulsing of antibiotics clearly demonstrate that in the last 24-hr period of cultivation antibiotics are ineffectual in concentrations which had markedly inhibited PFC when added at earlier time intervals. We interpret this to mean that as the young cells mature into immunocompetent cells, their functional capacity is less prone to antibiotic inhibition than is the earlier developmental cell. Agents which inhibit cycling cells are least effective at this time interval and, hence, we conclude that cell proliferation is not as necessary as in the two preceding phases.

Addition of antibiotics to cultures *in vitro* of antibody-producing cells has been studied before.[8–12] Ambrose[8] showed that actinomycin D in concentrations higher than 0·01 μM inhibited humoral antibody titers; in concentrations less than 0·01 μM, actinomycin D addition showed a transient increase in humoral antibody synthesis. He also showed experimental support that a repressor-like substance [antibody-inhibitory material (AIM)] is released during the productive phase of antibody synthesis and suggested that low levels of actinomycin D would inhibit the AIM and, hence, allow for continued antibody synthesis in the productive phase. Our experiments showed no stimulation of PFC with the addition of any of the three antibiotics at any temporal interval of the cultivation period. An interpretation consistent with the findings of Ambrose[8] and our laboratory is that, although there is no further increase in numbers of PFC, higher antibody titers may be attained from PFC already present by a selective suppression of AIM by actinomycin D.

Acknowledgement—I thank Vicente Llacer and Mrs. Lilly Loftfeld for their skillful technical assistance.

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