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Effect of antibiotics on antibody-forming cells *in vitro**

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PHYTOHEMAGGLUTININ, pokeweed mitogen, allogeneic cells and heterologous antisera[1–3] have been shown to transform cultured lymphocytic cells to a blastoid appearance. Parallel biochemical studies have indicated that these agents enhanced the incorporation of radioactive precursors into protein,[4] RNA,[5] and DNA[6–8] and that RNA and protein synthesis preceded DNA synthesis. The above studies have been viewed as useful experimental models for the study of early cytochemical events involved in antibody formation.

A more suitable biological test system to study antibody-forming cells would be a system *in vitro* in which a population of cultured cells would respond to antigen, undergo cell proliferation and mature into cells that are immunologically competent. Mishell and Dutton[9] have recently introduced a technique whereby dissociated cell suspensions obtained from spleens of normal mice were immunized *in vitro* to heterologous erythrocytes to produce hemolysin plaque-forming cells (HPFC). HPFC can be estimated by the technique of localized gel hemolysis introduced by Jerne *et al.*[10] Antibiotics, most of which are selective inhibitors of RNA, DNA or protein synthesis, may be used to advantage in dissecting the various phases of antibody production. This communication presents some preliminary experiments dealing with effective concentrations of various antibiotics which inhibit the production of antibody-forming cells *in vitro*.

Male C57L × A/He (LAF_1) mice between 8 and 16 weeks of age were obtained from Jackson Memorial Laboratories, Bar Harbor, Maine. Puromycin dihydrochloride, streptomycin sulfate and mitomycin C were obtained from Nutritional Biochemical Corp. Cycloheximide (Acti-dione) was supplied by Upjohn Company. Actinomycin D was supplied by Merck, Sharp & Dohme. Chloramphenicol was obtained from Parke, Davis & Company. Fetal calf serum, lyophilized guinea pig complement and sheep red blood cells were obtained from Colorado Serum Company. Concentrates of vitamins, essential amino acids, nonessential amino acids, glutamine and sodium pyruvate were obtained from Grand Island Biological Company. The cell culture conditions for the induction of HPFC *in vitro* were described by Mishell and Dutton.[9] Details of the procedure used to determine hemolysin plaques were reported by Jerne *et al.*[10] As a routine procedure, $1{\cdot}5 \times 10^7$ dispersed spleen cells in 1·0 ml of medium were planted with sheep red blood cells. Drugs were added on the second day and cells were harvested and analyzed for HPFC on the fourth day after planting. Two experiments were performed in replicate cultures for each drug concentration. Each experiment represented dispersed spleen cells pooled from five mice.

Studies on the effects of six antibiotics are shown in Table 1. The number in the second column for each drug concentration represents the average of two experiments. The measured effect at each drug concentration is expressed as per cent of control in the third column. Drug concentrations are given in terms of molarity (fourth column) and in terms of micrograms per milliliter of culture (last column).

Warner *et al.*[11] reported that in HeLa cultures protein synthesis was completely blocked at concentrations of cycloheximide (150 μg/ml) which did not affect ribosomal RNA synthesis or ribosome maturation. Smith *et al.*[12] have shown that cycloheximide at a concentration of 0·1 μg per ml reduced cell cultures to 50 per cent of control value. In our studies the concentration of cycloheximide employed ranged from 0·005 to 5 μg per ml of cultured spleen cells. At the lowest concentration employed, i.e. 0·005 μg per ml, the number of HPFC was inhibited to 5 per cent of control value.

Wust and Hanna[13] reported that actinomycin D was effective in suppressing antibody titers when injected into mice 4–8 hr prior to antigen injection and was not effective when injected prior to or after this period. Studies *in vivo* dealing with serum antibody titers may be experimental artifacts due to the delay with which serum levels reflect the more rapid alterations in antibody-forming cell populations. Uyeki and Klassen[14] showed that peak levels of developed antibody-forming cells appeared 1 week prior to peak serum levels of developed hemolysin. Thus, the precise mode of

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action of actinomycin D on antibody-forming cells may be obscured in such systems *in vivo*. In our dispersed spleen cell cultures *in vitro*, five different doses of actinomycin D ranging from 0·01 to 0·2 μg per ml were used. The results indicated that doses of 0·05–0·2 μg/ml inhibited HPFC to less than 5 per cent of control value. A dose of 0·01 μg/ml was shown to inhibit HPFC to 58 per cent of control value. On the basis of the low concentration of actinomycin D employed in our studies, it would appear that the inhibition of HPFC observed in our studies may be related to its effect on ribosomal maturation rather than to the inhibition of messenger RNA synthesis, which requires higher doses.[15]

Weisburger and Wolfe[16] demonstrated that chloramphenicol suppressed the primary antibody response against bovine gamma globulin in rabbits, but had no effect on the secondary response. They also noted that in a cell-free system chloramphenicol, at a concentration of 0·1 μM (3·2 μg/ml), inhibited protein synthesis in rabbit reticulocyte ribosomes. In our studies, chloramphenicol con-

TABLE 1. EFFECT OF ANTIBIOTICS ON SPLEEN HEMOLYSIN PLAQUE-FORMING CELLS *IN VITRO*

Drug	No. of HPFC per 10^6 cells planted*	% of control*	Final drug concentrations	
			(molarity × 10^{-6})	(μg/ml)
	197	control		
	10	<5	0·017	0·005
	13	<5	0·035	0·01
Cycloheximide	3	<5	0·350	0·10
(Acti-dione)	2	<5	1·700	0·50
	1	<5	3·500	1·00
	0	<5	17·000	5·00
	111	control		
	60	54	0·008	0·01
	38	34	0·016	0·02
Actinomycin D	4	<5	0·04	0·05
	1	<5	0·08	0·10
	0	<5	0·16	0·20
	53	control		
	73	137	0·1	0·032
	65	122	0·16	0·052
Chloramphenicol	60	113	0·57	0·18
	60	113	1·0	0·32
	54	102	1·6	0·52
	55	103	4·9	1·58
	60	113	10·0	3·23
	20	37	100·0	32·30
	231	control		
	139	60	0·1	0·06
Streptomycin	114	50	0·3	0·17
	73	32	1·0	0·58
	63	27	3·0	1·74
	15	<5	6·1	3·58
	83	control		
	43	51	0·03	0·01
Mitomycin C	17	20	0·30	0·10
	3	<5	3·00	1·00
	0	<5	30·00	10·00
	320	control		
	213	66	0·5	0·27
Puromycin	222	69	1·0	0·54
	3	<5	5·0	2·72
	0	<5	10·0	5·44

* Average of two experiments.

centrations which were equivalent to and higher than those employed by Weisburger and Wolfe did not inhibit the formation of antibody-producing cells. A concentration of 10^{-4}M (32 μg/ml) was required to inhibit the production of HPFC. Our studies indicated that the production of HPFC *in vitro* was relatively insensitive to chloramphenicol. The discrepancy between our finding and those of Weisburger and Wolfe is not understood at present.

Krueger[17] reported that streptomycin inhibited antibody synthesis in rabbit spleen and lymph node cultures at concentrations (200 μg/ml) which were not lethal and which produced no detectable morphological changes in cell cultures. In our studies, drug concentrations as low as 0·17 μg/ml (3×10^{-7}M) inhibited the number of HPFC to 50 per cent of control value.

The lethal effect of mitomycin C is associated with changes in DNA synthesis. Studies by Szybalski and Iyer[18] have indicated that mitomycin C cross-linked the DNA strands. Peterkofsky and Tomkins[19] demonstrated that mitomycin C (30 μg/ml) inhibited DNA synthesis in hepatoma cell cultures. In the concentrations employed in our studies, 0·01 μg/ml (3×10^{-8}M) inhibited HPFC to 50 per cent of control value.

In various microbial and mammalian cell systems, puromycin inhibits protein synthesis by inhibiting the transfer of amino acids into the protein molecule[20] or by inducing the release of nascent polypeptide from ribosomes.[21] Warner *et al.*[11] have shown that brief exposure to puromycin (150 μg/ml) resulted in a reduction of new ribosomes emerging from the cytoplasm of HeLa cell cultures. Our studies indicated that puromycin, at a concentration of 0·5 μg/ml, inhibited HPFC production to 66 per cent of control value. Doses higher than 1·0 μg/ml inhibited the production of antibody-forming cells to less than 5 per cent of control value.

Table 2 compares the ID_{50} dose (50 per cent inhibition) of various antibiotics on cell cultures of KB cells[22] with our study on HPFC *in vitro*. The comparison reveals that, with the exception of

TABLE 2. COMPARISON OF ID_{50} VALUES (50 PER CENT INHIBITION DOSE) ON KB CELL CULTURES[22] WITH HEMOLYSIN PLAQUE-FORMING CELLS *IN VITRO*

Antibiotic	Cytotoxicity of KB cells ID_{50} (μg/ml)*	HPFC *in vitro* ID_{50} (μg/ml)†
Cycloheximide	0·1	0·005
Chloramphenicol	25·0	25·0
Mitomycin C	0·025	0·01
Puromycin	—	1·5
Puromycin amino nucleoside	2·5	—
Streptomycin	>100·0	0·17
Actinomycin D	6×10^{-5}	0·01

* References 12 and 22. Antibiotics in contact with cells for 3 days; ID_{50} value calculated from log-dose response curve.

† Antibiotics in contact with cells for 2 days; ID_{50} is an approximate value needed to reduce HPFC to one-half that of untreated controls.

actinomycin D, the HPFC cultures *in vitro* were more sensitive to lower concentrations of antibiotics than were KB cell cultures. The ID_{50} actinomycin D dose of 5×10^{-5} μg per ml in the study of Smith *et al.*[22] is surprisingly low; our studies indicated an ID_{50} dose of actinomycin D to be in the range of 0·01 μg/ml. The reason for this discrepancy is not known.

In studies of established cell lines such as the KB strain of human epidermoid carcinoma cells, drug effects are assessed by a numerical tally of the surviving cell population. On the other hand, an added complexity of our assay system is that counted cells must be antigenically stimulated *in vitro* to produce a population of cells having a specialized function, viz. antibody production. Hence, our studies on short-lived cultures analyze but a small percentage of the total cells planted. A select population of cells, dependent on induction, proliferation and maturation for its expression as antibody-producing cells, may be more sensitive to antibiotics than a culture system involved in cell

proliferation alone. Since drugs were added 48 hr after planting, the present experiments exclude drug effects during the initial 48-hr period. To further define the loci of inhibition with respect to induction, proliferation and maturation of immunocompetent cells, we are currently examining antibiotic effects at various temporal intervals after cell culture.

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The effect of cycloheximide on ribonucleic acid and protein synthesis in rat liver*

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NUMEROUS carcinogens[1] and antitumor agents[2–4] produce alterations in the function and ultrastructure of the nucleolus, the site of synthesis and maturation of the ribosomal precursors.[5,6] Separation of nucleolar granules and fibrils into two or more distinct zones is designated as nucleolar segregation or nucleolar "cap" formation.

Cycloheximide produces nucleolar ultrastructural lesions in hepatic and pancreatic parenchymal cells of rats[7] and inhibits protein synthesis at the polysomal level,[8,9] but exerts no direct effect on

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