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MASTER

TECHNICAL PROGRESS REPORT

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AT(11-1)-1632

Mechanisms of Immunosuppression and Cellular Mechanisms of Secondary Disease

DISCLAIMER

Technical Progress Report - Contract AT(11-1)-1632
Kansas University Medical Center

Effort: E.M. Uyeki, Principal Investigator - 40% of time for 12 months is devoted to this project.

The Research Objectives of the proposal:

A) Mechanisms of immunosuppression by radiation and chemical agents.

B) To gain further knowledge of the basic cellular mechanisms of secondary disease.

Outline:

A-1. Antibiotic effects on the developmental phases of the immune response, in vitro.

A-2. Effect of anti-tumor agents on the developmental phases of the immune response, in vitro.

A-3. Variability in PFC production, in vitro, by spleen cells from normal animals.

A-4. Effect of stabilizers and labilizers on PFC production.

A-5. Studies on DNA-RNA hybridizations.

B-1. Studies on mixed lymphoid cell interactions using PFC as an indicator of cytotoxic potency. Allogeneic interaction of normal spleen cells.

B-2. Allogeneic interaction between effector and target cells.

A-1. Antibiotic effects on the developmental phases of the immune response, in vitro.

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 3 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, is 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 is 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.

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 (1,2), puromycin (3,4) and cycloheximide (4) 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 (24) similarly has used antibiotics as biochemical probes in his studies on ribosome biogenesis of HeLa cells.

We, as well as others (5,6,7), found the peak of PFC production occurred 4 days after planting. Nossal et al. (8) 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 (5), 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 to 24th hour of culture). Thus, daily additions (Figure 1) 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 (6,8,9) showed that at least two functionally different cell types in mouse spleen are required to initiate PFC production. Roseman (10) demonstrated that adherent cells (M) were resistant to radiation and that non-adherent cells (L) were extremely sensitive to radiation. It is noteworthy that the antibiotics, particularly actinomycin D, inhibited the contribution of L and M

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

cells to produce PFC in the reconstituted L and M experiments (Table 2). Collectively, Roseman's 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 play 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 (10), 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 24th and 72nd 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 (6) showed that the immune function of M cells is no longer needed after the first day of culture. At the other end, PFC's begin to appear on the 3rd day but reach peak levels on the 4th day. Cell proliferation is also ascribed to this period since agents which inhibit "cycling cells" are usually effective in suppressing production of PFC's (11,12).

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's 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 in vitro cultures of antibody producing cells has been studied before (13-17). Ambrose (13) 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 Ambrose's and our findings is that, although there is no further increase in numbers of PFC's, higher antibody titers may be attained from PFC's already present by a selective suppression of AIM by actinomycin D. (For a more thorough discussion, a pre-print of the paper submitted to Biochemical Pharmacology is included in this report).

A-2. Effect of anti-tumor agents on the developmental phases of the immune response, in vitro.

Some unpublished studies have been initiated on the effect of selected anti-tumor agents on the in vitro plaque forming cells. Agents chosen for this study were 6- mercaptopurine, amethopterin and vinblastine. ~~Results of these studies are included in~~ In general, the results of these studies have shown a similar pattern of inhibition; that is, the drugs are most effective when they are added to cultures on the initial day of plant and became less effective when added at later time intervals. Studies will be conducted on the effects of these agents on L and M cells.

A-3. Variability in PFC production, in vitro by spleen cells from normal animals.

Having performed the short-term cultures of PFC over the past four years, I have been impressed with the sensitivity and lability of the system. I should also like to note, that because of its sensitivity and lability, a considerable portion of our experiments (circa 20%) are rejected because of inadequate production of PFC's per million spleen cells. We usually reject experiments which have less than 60 PFC per 10^6 spleen cells planted. We have looked at experimental manipulations which may alter the response of spleen cells from normal animals. We noted that when spleen cells from different strains of mice were planted, each strain had a characteristic cell number which would give optimal amounts of PFC per million spleen cells planted. We planted normal spleen cells from various mouse strains in the following amounts: 0.5, 1.0, 1.5 and 2.0 x 10^7 cells. Cells were then incubated in the presence of SRBC for a period of four days and analyzed for PFC. The results of these studies are shown in figure 2. They were somewhat surprising. Having done most of our studies on the LAF_1 strain of mice, we expected increasing amounts of cells planted to give us a plateau. This was the response that we observed on LAF_1 (a). On the basis of efficiency of PFC produced per 10^7 cells planted, an optimum was obtained with 1 x 10^7 cells (e). However, the three other strains which we tested did not show the plateau effect; instead, an optimum was reached at 1.0 or 1.5 x 10^7 cells and the total numbers of PFC produced per dish actually diminished even though greater numbers of cells were planted. We have assessed cell viability at the various cell concentrations and found no differences in cell viability. Hence, we conclude that, in the in vitro culture performed in 35 mm tissue culture petri

dishes, it is important to find cell concentration optima. Perhaps, the simplest explanation for this surprising finding is to suggest that increased cell density of any given strain of mice results in a change in "signals" of relevant precursor cells to "turn off" the production of PFC. An alternative speculation would be that the increased cell density would be a "signal" to change to another kind of "end-cell stage" of terminal cells, the experimental assessment of which we do not currently possess. Hence, it would be important for us to find other assays for immunocompetent cells. (Perhaps rosette-forming cells, or indirect plaque forming cells).

A-4. Effect of stabilizers and labilizers on PFC production.

Some studies initiated by Dr. Garry have indicated a marked dependence of antigenicity on the intactness of SRBC membrane. He has utilized chlorpromazine, which has a biphasic action on the red cell membrane, to demonstrate this dependence. At high concentrations, ca. 10^{-3}M, chlorpromazine will lyse red cell membranes; at lower concentrations, 10^{-4} through 10^{-6}M, chlorpromazine has a stabilizing action on red cell membranes assessed by its ability to counter-act mild hypotonicity. He then utilized labilized and stabilized chlorpromazine-treated red cells and found that labilized or lysed RBC had no stimulatory activity, whereas chlorpromazine-treated red cells (in the concentration range of 10^{-4} to 10^{-6}M) had a stimulatory effect on PFC (about 150 percent of controls). At concentrations below 10^{-6}M, chlorpromazine-treated red cells were not significantly different from control RBC. This work is being prepared for publication in Biochemical Pharmacology.

He has also studied the effect of detergents on PFC, in vitro and found marked differences in the ability of detergents to inhibit the production of PFC. An anionic detergent, sodium dodecyl sulfate, was effective in suppressing PFC at 100 ppm; Triton-X 100, a non-ionic detergent was effective at 10 ppm, and cetyl trimethyl ammonium bromide, a cationic detergent, was effective in suppressing PFC production at 0.001 ppm when the detergents were added on the first day of culture. Hence, he observed wide concentration differences in the ability of different classes of detergents to suppress the formation of antibody producing cells.

A-5. Studies on DNA-RNA hybridizations.

Some preliminary attempts were made to hybridize cytological preparations of splenic cultures with radioactive RNA from splenic cultures. Although we were able to isolate RNA with high specific activity, we have not been successful in demonstrating cell-labeled RNA hybridization techniques using the method of Pardue and Gall (Proc. Nat. Acad. Sci. Vol 64: 600, 1969). Reports in the current literature indicate that this technique can be accomplished on certain tissue preparations (Chromosoma Vol 29: 268, 1970; Cytological localization of DNA complementary to ribosomal RNA in polythene chromosome of Diptera). It has become apparent, at least in my laboratory, that a great deal more developmental studies are necessary in mammalian cells before we can utilize this technique. I will opt to work on other areas which are

more currently fruitful. We will however, utilize the biochemical technique of Gillespie and Speigelmann (J. Molec. Biol. Vol 12: 829, 1965) to determine and further characterize the RNA that is inhibited by actinomycin D in the M fraction (see A-1 for discussion).

B. To gain further knowledge of the basic cellular mechanisms of secondary disease. I think we have gained insights into the mechanisms of secondary disease by studying mixed lymphocyte interaction coupled to the in vitro production of PFC - hereby shortened to MLI-PFC).

B-1. Studies on mixed lymphoid cell interactions using PFC as an indicator of cytotoxic potency. Allogeneic interaction of normal spleen cells.

Studies initiated by Hirano and Uyeki has just been published in Journal of Immunology and will be included in this report. By using the in vitro production of PFC as an indicator of cytotoxic potency, allogeneic spleen cell interactions were investigated. Dispersed spleen cells from two allogeneic mouse strains were mixed at the beginning of the culture period. The rationale for assessing interaction was as follows: If spleen cells from A and B animals are mixed and there is no interaction, the production of PFC in mixed cultures should be equivalent to the sum of PFC production by A and B cells cultured separately. On the other hand, if there is some interaction between the two allogeneic cell types, PFC's produced by the mixed cell culture should be different than the sum of cultures incubated separately. These studies may be summarized: A wide range of cell interactions was observed. Although considerable variations was noted from experiment to experiment, the results indicated that numbers of PFC produced was associated with the degree of histoincompatibility (H-2) of splenic lymphoid cells cultured together. In combinations which demonstrated an inhibitory effect on PFC production, these strains were generally strongly incompatible; on the other hand, lesser suppression of PFC formation or sometimes stimulation of PFC were associated with strain combinations of weak incompatibilities. We tentatively concluded that allogeneic interaction may affect all phases of the immune response to SRBC. However, our unpublished evidence favors the notion that cell interactions in the initial 24 hour culture period is the temporally important period to allogeneic interaction.

B-2. Allogeneic interaction between effector and target cells.

Many studies have shown cell-mediated cytotoxicity of sensitized cells (effector cells) for various "target" cells. These studies have pointed out characteristics of cell mediated cytotoxic effects that differ from those induced by humoral antibodies: 1) cell mediated cytotoxicity requires a longer time to develop. 2) initial contact between effector and donor cells is necessary, and 3) lysis of target cells by effector cells does not require complement. Suppression of PFC production in mixed cultures by effector cells had the following characteristics: 1) marked suppression of PFC production occurred only

when effector cells were combined with specific donor cells and that this cytotoxic action of effector cells appeared earlier than humoral antibodies in mice sensitized against target lymphoid cells. 2) initial cell-cell contact was necessary to demonstrate cytotoxicity and 3) factor (s) responsible for suppression of PFC production did not depend on the action of complement.

The studies are being extended and some of the current unpublished studies indicate the following: we used A/J strains as the sensitized animals (i.e., from which we obtained the effector cells) and C57Bl/6J served as the target and donor strain. A/J animals were immunized with an intraperitoneal injection of 5×10^7 donor strain cells. As early as four days after immunization, spleen cells from sensitized animals (A/J) were placed in culture with target C57Bl/6J cells. In a ratio of 1:2:: effector cells: target cells, PFC production was markedly inhibited. Hence, this study demonstrates that cell-mediated cytotoxicity can be obtained in a relatively short period of time and time-wise, is comparable to the production of antibody producing cells (PFC). Another finding which is of some interest is that when allogeneic cells are mixed together, the PFC suppressant activity is greatest when they are mixed together on the day of plant. When the effector cell is allowed to come in contact on Day 1 or later, the suppressant action is lacking or, sometimes, a stimulation of PFC is found. This study is in the preliminary stages and is being continued.

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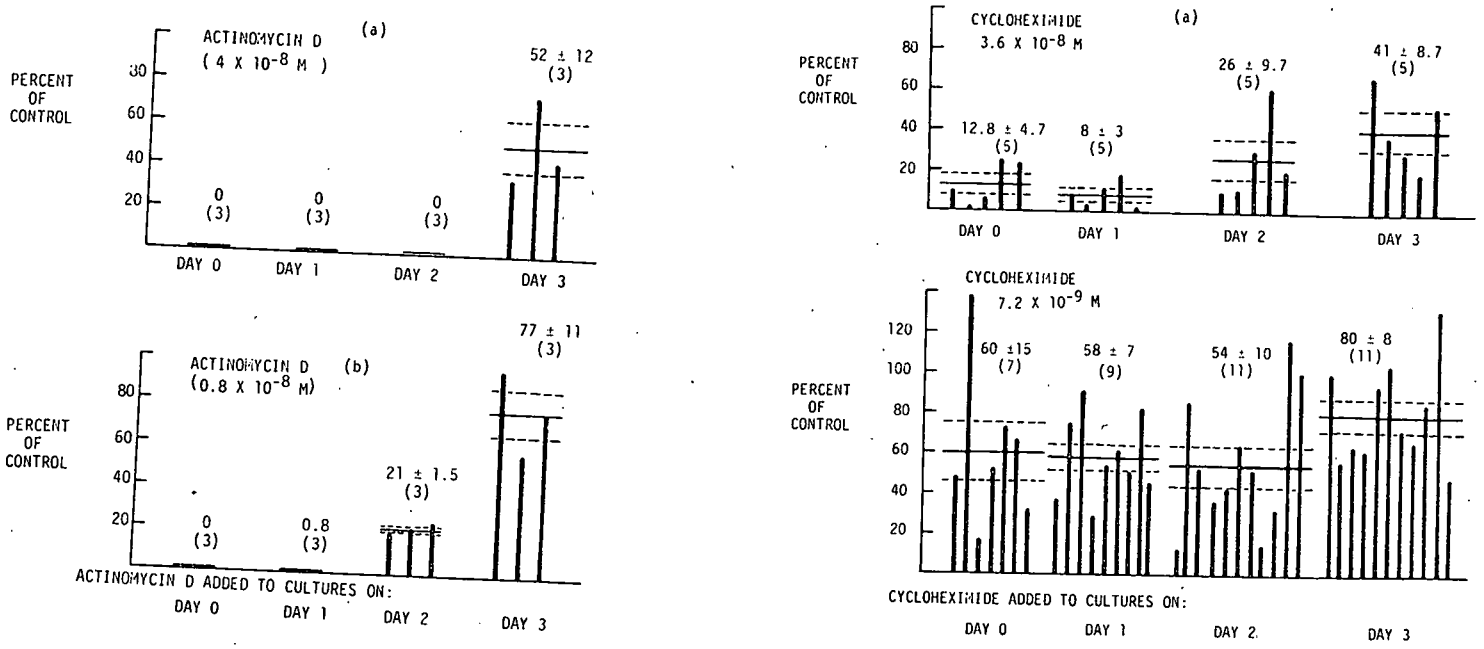
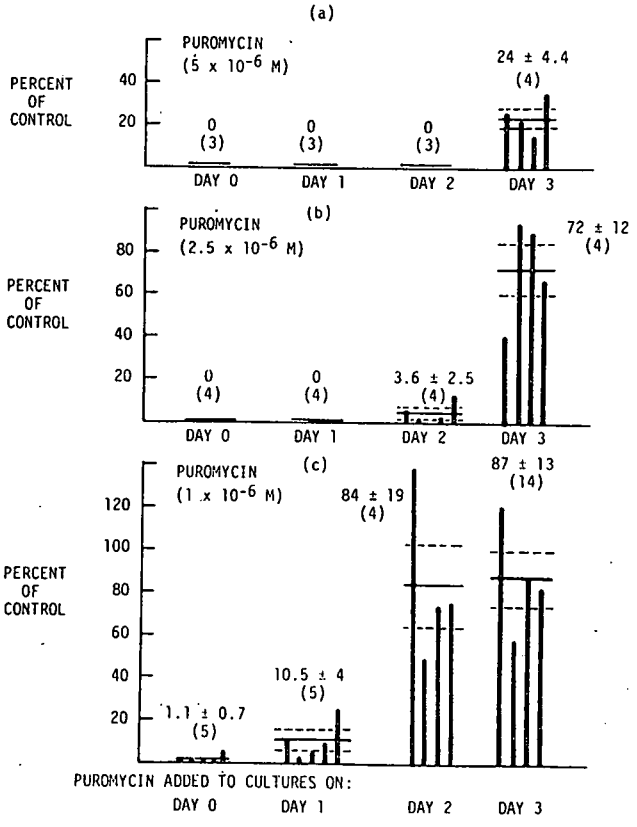
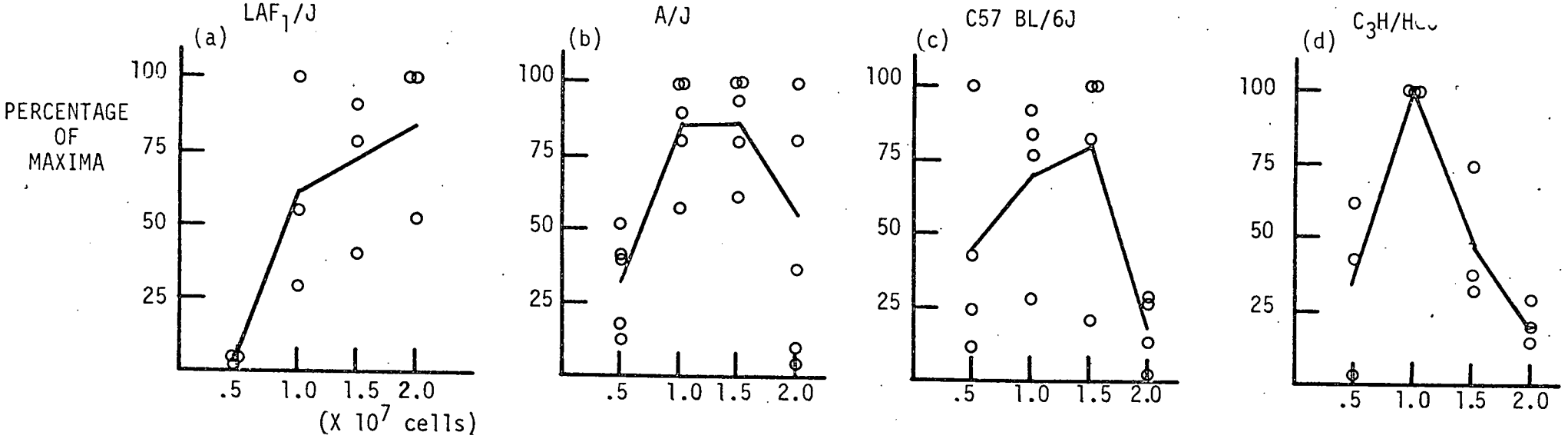
Figure 1


(a)
ACTINOMYCIN D
(4 X 10-8 M)
PERCENT OF CONTROL
0 (3)
0 (3)
0 (3)
52 ± 12 (3)
DAY 0
DAY 1
DAY 2
DAY 3
(b)
ACTINOMYCIN D
(0.8 X 10-8 M)
0 (3)
0.8 (3)
21 ± 1.5 (3)
77 ± 11 (3)
ACTINOMYCIN D ADDED TO CULTURES ON:
(a)
CYCLOHEXIMIDE
3.6 X 10-8 M
12.8 ± 4.7 (5)
8 ± 3 (5)
26 ± 9.7 (5)
41 ± 8.7 (5)
(b)
CYCLOHEXIMIDE
7.2 X 10-9 M
60 ±15 (7)
58 ± 7 (9)
54 ± 10 (11)
80 ± 8 (11)
CYCLOHEXIMIDE ADDED TO CULTURES ON:


(a)
PUROMYCIN
(5 x 10-6 M)
PERCENT OF CONTROL
0 (3)
0 (3)
0 (3)
24 ± 4.4 (4)
DAY 0
DAY 1
DAY 2
DAY 3
(b)
PUROMYCIN
(2.5 x 10-6 M)
0 (4)
0 (4)
3.6 ± 2.5 (4)
72 ± 12 (4)
(c)
PUROMYCIN
(1 x 10-6 M)
1.1 ± 0.7 (5)
10.5 ± 4 (5)
84 ± 19 (4)
87 ± 13 (14)
PUROMYCIN ADDED TO CULTURES ON:

CALCULATED ON THE BASIS OF PFC PER DISH



CALCULATED ON THE BASIS OF PFC PER 10^7 CELLS

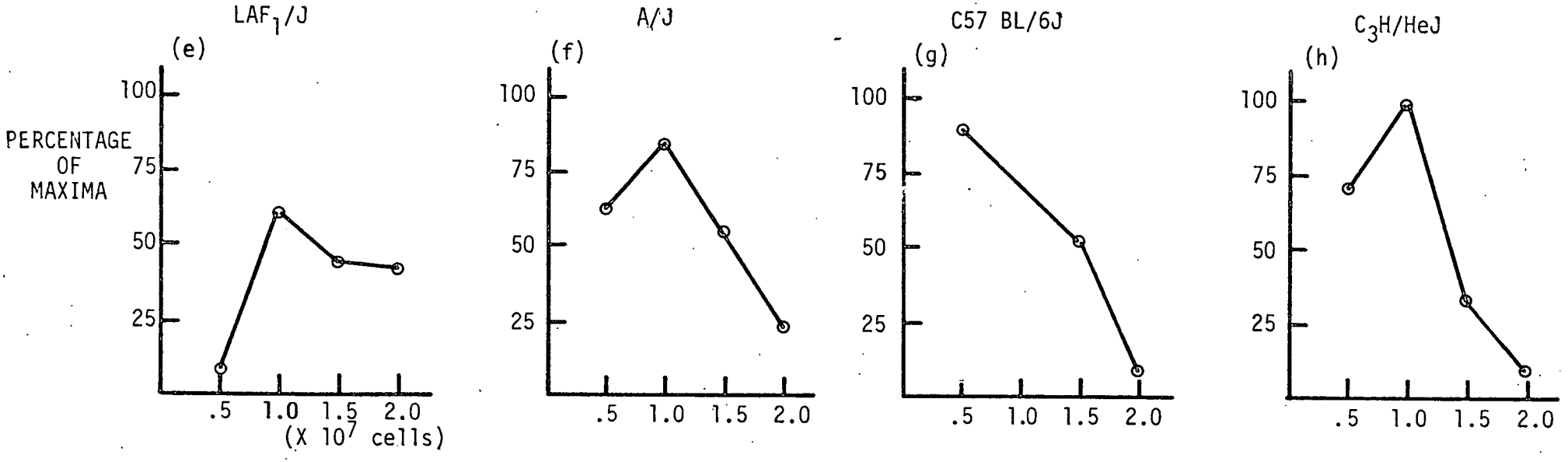


Figure 2

Table 1. Effects of a 40-minute 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 3 experiments

† Average of 5 experiments

Table 2. Effect of a 40-minute 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 5 individual experiments in which 3 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	Concentration µg/ml	Scheme "A" cpm/disk+ Experiment No. 1	Scheme "A" cpm/disk+ Experiment No. 2	Scheme "A" Percentage of control (average of 2 experiments)	Scheme "B" cpm/disk+ Experiment No. 1	Scheme "B" cpm/disk+ Experiment No. 2	Scheme "B" Percentage of control (average of 2 experiments)
L Cells	Control	--	3746	930	--	1920	3815	--
	Actinomycin D	0.02 0.05	3135 3365	999 980	95 97	1205 929	2900 2118	69 52
	Cycloheximide	1.5 15.0	2268 1868	999 312	84 41	1270 406	2283 597	62 19
	Puromycin	1.5 15.0	2155 1014	619 218	61 25	378 115	874 95	22 4
M Cells	Control	--	1340	340	--	2550	4440	--
	Actinomycin D	0.02 0.05	440 605	273 269	58 63	724 523	916 740	25 18
	Cycloheximide	1.5 15.0	257 177	168 63	35 16	418 182	441 194	13 5
	Puromycin	1.5 15.0	267 121	129 101	30 20	162 65	267 71	6 1

* Two microcuries of tritiated RNA precursors were added to control and drug-treated cells. The specific activity of the compounds were the following: 8-^{3}H adenosine (26 C/M), 8-^{3}H guanosine (4.7 C/M), 5-^{3}H cytidine (6.0 C/M) and 5-^{3}H uridine (4.0 C/M). Scheme "A" represents 1 hr pre-incubation with drug before a 4-hr pulse with tritiated RNA precursors. Scheme "B" represents 18 hrs pre-incubation 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.