AN ABSTRACT OF THE THESIS OF

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Title: THE EFFECTS OF ACTINOMYCIN D ON THE PRIMARY AND SECONDARY IMMUNE RESPONSES INDUCED IN VITRO

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Abstract approved: _____
Robert W. Newburgh

In order to assess the importance of RNA synthesis in the induction and antibody-producing phases of immune responses, actinomycin D was used to inhibit the primary and secondary in vitro immune responses of mouse spleen cells against heterologous erythrocytes. The presence of actinomycin D (0.1 or 0.125 μg/ml) throughout the entire four day culture period inhibited the development of plaque-forming cells (PFCs) in both primary and secondary responses by more than 99%. The same drug concentration, if added after 1, 2, or 3 days of culture, inhibited the number of PFCs on the fourth day by 81-99%.

Utilizing cultures of spleen cells from mice already undergoing a secondary immune response, no stimulation of the PFC number upon a 24 hour treatment with actinomycin D (1.25 μg/ml) was found, unlike the observation of Harris (J. Experimental Medicine 127:675, 1968), with a similar system of cultured rabbit spleen cells. The

treatment of these mouse spleen suspensions for 24 hours with actinomycin D (1.25 μg/ml) usually brought about a 90-95% decrease in the number of PFCs.

For both the primary and secondary in vitro immune responses the sensitivity to actinomycin D treatment throughout the culture period was determined. A drug concentration of 10^{-3} μg/ml caused no inhibition in either type of response, but rather caused some stimulation of PFC numbers by the end of the fourth day of culture. The secondary response was inhibited by 43% and 98% by actinomycin D concentrations of $3x10^{-3}$ and 10^{-2} μg/ml respectively. The primary response was inhibited by 95% and 99% by actinomycin D concentrations of $3x10^{-3}$ and 10^{-2} μg/ml respectively. This sensitivity of the entire response is much greater than that reported by Uyeki and Llacer (Biochemical Pharmacology 18:948, 1969) for actinomycin D additions at a later stage of the response.

The spleen cell suspension was separated into a macrophage-rich fraction and a lymphocyte-rich fraction. Treatment of the macrophage population for one hour with actinomycin D (0.1 μg/ml), either during or before the addition of antigen, inhibited by 95% the immune response which resulted following removal of the drug and addition of the lymphocyte-rich population to reconstitute the original cell suspension.

The results are discussed with respect to the possible events occurring during the response which would account for the observed

sensitivity to actinomycin D. A suggestion is made that the extremely high sensitivity of the early phases of the immune response may be due to the need for synthesis of some very large RNA molecule.

The Effects of Actinomycin D on the Primary and Secondary Immune Responses Induced *In Vitro*

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

THE EFFECTS OF ACTINOMYCIN D ON THE PRIMARY AND SECONDARY IMMUNE RESPONSES INDUCED IN VITRO

INTRODUCTION

The mode of synthesis of DNA, RNA, and proteins, and the relation between the transfer of genetic information from DNA to RNA and thence to protein were developed in the 1950's and early 1960's from experiments primarily with bacterial and viral systems, and only occasionally higher organisms. The "dogmas" of molecular biology thus determined were often assumed to be equally valid in the higher organisms. In many aspects this conclusion was substantiated with experimental evidence. However, some definite differences between the two systems are evident. For example, in the bacterial system the concept of messenger RNA was developed around a rapidly labeled or rapidly turned-over RNA fraction(7,31) and it was assumed to have a short half-life. This resulted in the idea that a positive control of protein synthesis could be brought about by simply controlling the rate of synthesis of the appropriate messenger RNA molecules. In mammalian systems, however, the observation was made that in some cases messenger RNA molecules continue to act in protein synthesis long after RNA synthesis is no longer possible or is severely inhibited; i.e. the reticulocyte hemoglobin synthesis system (58), the synthesis in chick embryo of feather proteins (5),

and of lens proteins (59), some mitochondrial enzymes (17), immunoglobulin synthesis (43) and others.

The Study of Differentiating Systems

To elucidate the differentiation process of higher organisms at a molecular level, it is of vital importance to determine the degree to which extrapolation of bacterial mechanisms are useful to mechanisms extant in the higher organisms. One area in which further clarification is needed is the temporal relationship between messenger RNA formation and the phenotypic expression of the results of that message. Several criteria can be established for a suitable system in which to study this temporal relationship. First, the system should involve the induction of synthesis of a particular, well-defined protein and/or mRNA molecule. In this case either natural induction processes such as occur in embryonic development or other specific differentiation systems could be used; or (and perhaps preferably) a system could be devised which involves experimental induction, so that the time of onset of the induction process could be determined with comparative accuracy. The second criteria is that the product synthesized should be capable of specific detection in small amounts in the presence of large excesses of other similar molecules. In this case the production of specific enzymes or antibodies, whose biological specificity permits their detection,

are good possibilities. The third criteria for the system would be that the environmental conditions and the genotype of the tissues be strictly defined when the induction process occurs from one experiment to the next. Tissue culture systems provide the necessary control of the environment and also allow the tissues from one animal or group of animals to serve for several replicate experiments.

The system used in this work, the induction of a specific immune response in vitro, provides a good fit with these criteria. In some in vivo experiments RNA synthesis did not appear vital for the secondary response to occur (26). For this reason it was deemed of prime importance that the primary response be included in some of the in vitro work.

The history of attempts to demonstrate the production of antibodies in vitro is nearly as old as tissue culture itself. In 1912 Ludke developed a method of culturing spleen and bone marrow from immunized rabbits and guinea pigs and after five days in culture agglutinins (agglutinating antibodies) could be demonstrated in an extract of the tissue. Salle and Mc Omie (56) have summarized this work and the work of many others who used this method successfully. However none of the induction processes occurred during the culture period.

The first report of a successful in vitro induction of an immune response in cultured tissues of unimmunized animals was made by

Carrell and Ingebrigsten in 1912 (13). By introducing goat erythrocytes to cultures of normal guinea pig lymph node and bone marrow tissue, they were able to demonstrate the induction of hemolysins (hemolytic antibodies) against the goat erythrocytes. Although this method is basically the same as the method used in the experiments described herein, many authors attempted to reproduce these experiments without success (56). It was not until 1965 that a positive primary response was induced by Globerson and Auerbach (37) in tissue culture by the simple addition of antigen to cultures which had been stimulated to cell division by the addition of phytohemagglutinin. Saunders and King (57) used the sensitive assay of phage inactivation to demonstrate synthesis of anti-phage antibodies in a combined spleen-thymus organ culture system.

One reason for the resurgence of the hemolysin-producing system for the study of the induction process was the development by Jerne and his coworkers of a method of detecting single cells producing specific hemolysins (hemolytic plaque-forming-cells or HPFC, or more commonly PFC)(37). A description of an adaptation of this method is found in the methods section. A similar method for detecting single antibody producing cells using a gum of carboxymethylcellulose (CMC) in place of agar as the immobilizing medium was independently developed by Ingraham and Bussard (36).

Several classes of antibodies are produced in the <u>in vivo</u>

immune responses. In the first few days after antigen has been introduced the antibodies are of the γM type with a molecular size of 19S as determined by ultracentrifugation experiments (69). This is the type of antibody production which is identified by the hemolytic plaque formation techniques of Jerne and Nordin (38) and that of Ingraham and Bussard (36). A few days later in the course of the response it is found that the number of cells producing 19S antibodies has dropped and a much larger number of cells are now producing the γG type antibody which is a 7S molecule. The Jerne type of assay system has been adapted to demonstrate the presence of 7S antibody forming cells by Sterzl and Riha (63) and by Dresser and Wortis (18). This method involves treating the plaque-forming cell assay plates with antiserum directed against γG globulins before the addition of complement. For most of the following work only the 19S plaque-forming cells have been detected because only the first few days of the response are examined.

Using the same conditions for short term culture as for the assay procedure, Bussard was able to demonstrate the induction of a primary immune response in normal mouse peritoneal cells after a lag period of only 20 hours (10). The outstanding characteristics of this system are that: a) the cells are not cultured in liquid media, but in a gum of CMC containing both the peritoneal cells and the red cell antigen, b) the CMC has adjuvant properties when studied in vivo

and may have a similar enhancing effect in this in vitro system, c) peritoneal cells were used in the hope that they would survive the various processes involved in preparing the slides since they were accustomed to living as free and independent cells, unlike spleen cells which failed in this system to produce plaque-forming cells. The plaques formed in this system were complement dependent, dependent on viable cells, inhibited to some extent by actinomycin D, and the total number of plaque forming cells dependent on the concentration of peritoneal exudate cells in such a way as to approximate a second order curve, possibly indicating that more than one cell is involved in the sequence of events leading to a true de novo immunization of normal cells.

Mishell and Dutton (48) developed the system for the immunization in tissue culture of dissociated mouse spleen cells, which formed the basis for the experiments described herein. The salient points of this method are: a) a relatively high cell concentration in liquid medium, b) the use of a "nutritional cocktail" to promote longevity of the cells, c) a lower than usual oxygen concentration, d) the use of a rotary shaker (later replaced by a rocker table) and e) the inclusion of fetal bovine serum in the liquid media. The in vitro system gave PFC numbers only about one $\log_2$ less than those seen in vivo. Increases of plaque-forming cells up to 1000 times the background were observed.

Several variations of this basic culture method have since been used in attempts to elucidate the cellular interactions involved in the immune induction. The most pertinent of these studies is the work of Mosier (50), who separated the spleen cell suspension into two cell populations according to the cell's ability or disability to adhere to the surface of the plastic culture dish. The cell population adhering to the culture dish was rich in macrophage-like cells. The non-adherent population was rich in lymphocytic cells. Neither of these two cell populations alone could undergo an immune induction in the culture system of Mishell and Dutton, but when the two populations were recombined a normal immune induction could be produced. This demonstrated the need for at least two cell types which co-opeate in the immune induction. By varying the relative amounts of the two cell populations in the culture system, and observing the resultant changes in the induction kinetics, it was determined that three cell types were needed in a co-operative effort for the complete induction process (52). Mosier was also able to demonstrate the formation of specific clustering of the cells during the incubation period (49). If the formation of these clusters was blocked (by the removal of the rocking action during the incubation period), or if the clusters were formed and then disrupted by agitation and their reformation blocked, the induction process was severely inhibited.

The properties of the antibiotic actinomycin have recently been

reviewed in a monograph edited by Salman Waksman (71), in whose laboratory the drug was first isolated. The structure of actinomycin D (see Figure 1) was determined by Bullock and Johnson in 1957 (9). All of the actinomycins have the same chromophore but differ in the cyclic amino acid side chains. Actinomycin D is identical to actinomycin C_1 which was isolated from a different Streptomyces species (8). Actinomycin C_2 differs from actinomycin D by having an Aileu (D-alloisoleucine) residue substituted for valine in one of the two side chains, and actinomycin C_3 has Aileu substituted for both of the valyl moieties. The commercial actinomycin preparations used in most biochemical research reported in the literature are Dactinomycin (actinomycin D) and Cactinomycin (actinomycin C) which is a mixture of 10% actinomycin D, 45% actinomycin C_2 and 45% actinomycin C_3.

Actinomycin D is a strong inhibitor of DNA-dependent RNA synthesis in intact cells, but does not inhibit RNA-dependent RNA synthesis (RNA-virus replication), nor does it inhibit cellular DNA synthesis at a concentration of 0.1 μM which does inhibit the RNA synthesis (55). In an examination of the effect of actinomycin D on the individual enzymatic reactions in cell free systems, Hurwitz et al. demonstrated that both RNA-polymerase (DNA-dependent) and DNA polymerase were inhibited to some extent by actinomycin D, but the DNA-polymerase was about ten-fold less sensitive to the actions of the drug than was the RNA polymerase (35). This inhibition is

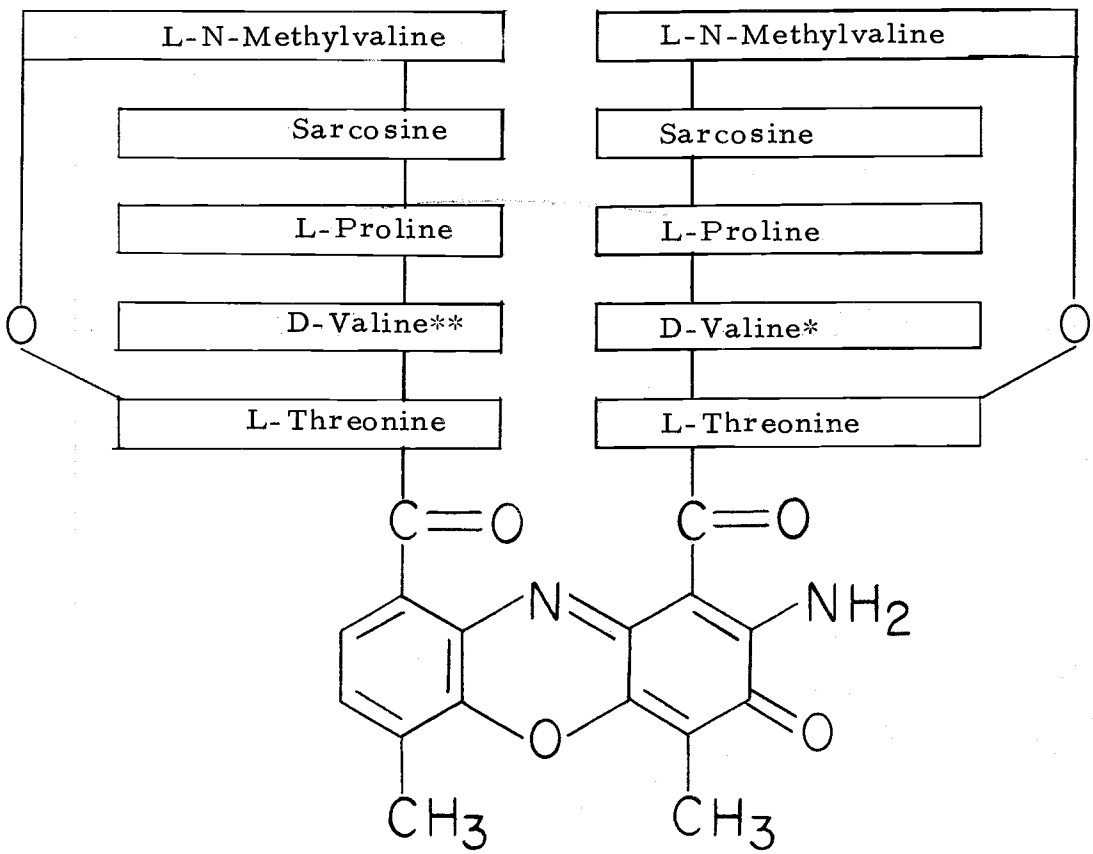


Figure 1. Chemical structure of actinomycin D and actinomycins C_2 and C_3.

actinomycin D (C_1) . . . shown above
actinomycin C_2 . . . . . D-alloisoleucine substituted for valine*
actinomycin C_3 . . . . . D-alloisoleucine substituted for both valine* and valine**

brought about by the selective binding of the actinomycin D molecules to the deoxyguanosine residues of the DNA in its native helical configuration. This DNA-actinomycin D (complex) does not serve as an efficient template for the function of RNA polymerase (28).

MATERIALS AND METHODS

Biological Supplies

Animals

The mice used for most of the experiments were BDF_1 purchased at the age of eight to ten weeks from Simonsen Laboratories (Gilroy, California) and from the Jackson Laboratory (Bar Harbor, Maine). This strain is the first generation progeny from a cross between the highly inbred lines C57BL/6J (female) and DBA/2J (male). These mice have the advantages of genetic uniformity combined with an increased resistance to disease and greater longevity common to hybrids as compared to inbred mice (30). This same strain of mice was used by Mishell and Dutton (47) and by Mosier (50) in their work with primary immune responses in vitro. The animals were kept in polypropylene cages (Aloe #VMP 25) and given Purina rat and mouse chow and water ad libitum.

Antigen

The sheep blood used in these experiments as the antigen was collected in Alsever's solution (Microbiological Associates) from sheep kept at Oregon State University for such purposes.

Chemicals

Media

Ingredients for the tissue culture media were purchased from Microbiological Associates (Albany, California) and from Grand Island Biological Co. (Oakland, California). The fetal bovine serum for the early experiments was purchased from Microbiological Associates; however, most of these experiments did not provide the correct conditions for the *in vitro* initiation of the immune response. For the remainder of the work, fetal bovine serum was purchased from Colorado Serum Company Laboratories (Denver, Colorado). This serum, especially Lot #280 provided a much improved environment for the initiation of the immune response *in vitro*. The culture media and balanced salt solutions used in this thesis are shown in Table 1.

Actinomycin D

The actinomycin D was the generous gift of Dr. Norman G. Brink, Director of Bioorganic Chemistry, Marck Sharpe and Dohme Research Laboratory at Rahway, New Jersey.

Table 1. Media

Abbreviation	Use	Preparation
MEM suppl. + 5% FBS	Culture medium	Eagle's minimal essential medium - modified for suspension culture - supplemented with Eagle's non-essential amino acids, 100 mM sodium pyruvate, and 5% fetal bovine serum.
MEM without bicarbonate	Preparation of original cell suspension, suspending cells for PFC assay, dissolving agarose for PFC assay.	Eagle's minimal essential medium - modified for suspension culture. Made up from 10x concentrate in pyrogen-free distilled water. The pH was adjusted to neutrality with 0.3 N NaOH.
Nutrient additive	Feeding of cultures	2x concentrated Earle's minimal essential medium - modified for suspension culture - supplemented with 2x Eagle's non-essential amino acids, 200 mM sodium pyruvate. This is mixed with an equal amount of fetal bovine serum before feeding to cultures.

Gas Mixture

The tanks of gas mixture (C_2 -7%, CO_2 -10%, and N_2 -83%) were purchased from Salem Welding Supply Co. (Salem, Oregon). The gas was humidified by bubbling it through two bottles of 20% Copper Sulfate in water held at 37°C before introducing to the incubation chamber.

Complement

The complement was ordered from several sources, such as Difco and Grand Island Biological Co. in the form of lyophilized guinea pig serum. The complement purchased from Gibco was reconstituted with water instead of the solution supplied by the manufacturer because the solution contained sodium azide as a preservative and this may cause an inhibition of the cell's ability to produce antibodies. The azide would not interfere in the original Jerne plaque-forming cell assay because the complement was added to the petri dishes only at the end of the incubation period, and thus after the antibodies necessary for the assay had been produced. However in the slide method of PFC determination used in this thesis the slides were exposed to the complement (and thus the azide if present) for the entire incubation period. The reconstituted complement was absorbed twice with washed sheep RBC, centrifuged in the clinical centrifuge and then

frozen in aliquots which would provide enough complement to incubate with one tray (8 or 11 slides).

Agarose

The agarose used in the plaque-forming cell determinations was purchased from two sources; that used in the early experiments was purchased from Bio-Rad Laboratories (Richmond, California) Lot #8390; that used in the later experiments was purchased from L'Industrie Biologique Francaise (Genvilliers, France) from a lot shown to be free from any activity against complement.

Preparation of Spleen Cell Cultures

The spleen cell cultures were prepared essentially by the method of Mishell and Dutton as modified by Mosier (50). The mice were killed by cervical dislocation and the fur was washed with 70% ethanol. Using sterile techniques, an incision was made through the peritoneum and the spleen was dissected out with small blunt forceps. At this point the fatty tissue and some of the membranes surrounding the spleen were removed from the surface. The spleen was then placed into ice cold media (MEM without bicarbonate, pH adjusted to about 7.2 with 0.3N NaOH). Since the MEM used was made for spinner cultures and thus had ten times the phosphate of the original MEM formulation, this media still contained some buffering capacity.

Usually five or six spleens were dissected out at one time and put into the sterile petri dish containing about 12 ml of the cold media. The petri dish was then placed in a sterile hood and two sterile sharp-pointed forceps were used to gently tear apart the splenic capsule and mince the tissue into very small chunks, releasing the free cells into the medium. This operation usually took from 10 to 15 minutes. A sterile pasteur pipette was then used to aspirate the cell suspension several times to break up the loose cell aggregates. The media and tissue fragments were then transferred using the pasteur pipette to a sterile 12 ml centrifuge tube in an ice bucket and allowed to stand for five minutes to allow the heavy tissue chunks to settle to the bottom. The supernatant suspension of mostly single cells was then transferred with another sterile pasteur pipette to a second centrifuge tube (30 ml capacity) in the cold and the suspension was centrifuged at room temperature for ten minutes at 1800 rpm (600 x g) in a Sorvall table model centrifuge with a swinging bucket head. The supernatant was drawn off and the cell pellet suspended in 10 ml of the complete culture medium (MEM supplemented + 5% or sometimes 10% fetal calf serum) with a vortex mixer. Appropriate dilutions of this suspension were then made, and the cultures prepared by pipetting 1.0 ml of cell suspension into each culture dish. The culture dishes were 35 mm x 10 mm round plastic tissue culture dishes #3001 of Falcon Plastics (Oxnard, California). Inhibitors and/or

antigen (SRBC) were added either at this point or added to the dishes immediately prior to the introduction of the cell suspension. These substances were added using an Eppendorf automatic 5 μl pipette and a sterile plastic tip. The sterile tip was the only portion of the Eppendorf apparatus that touched the liquid.

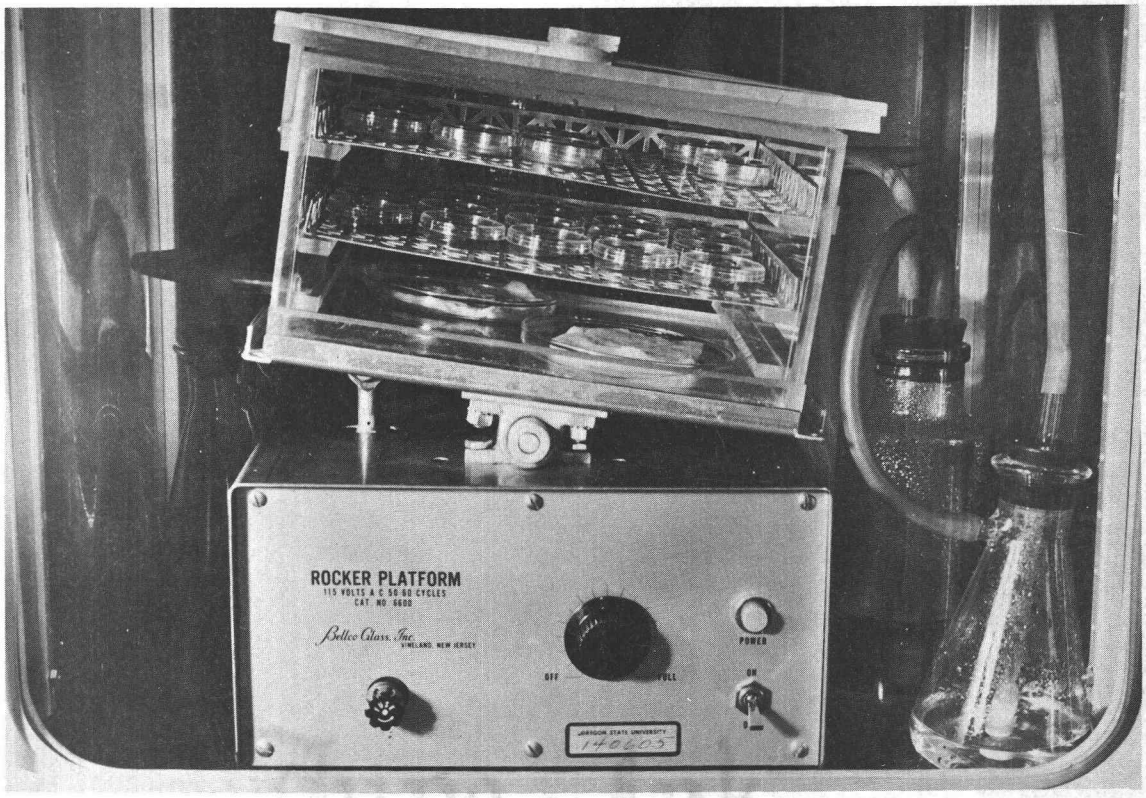
Incubation and Feeding of the Cultures

The incubation equipment is shown in Plate 1. A small chamber for incubating several trays of cultures in the controlled environment was constructed from plexiglass. The premixed atmosphere of 7% O_2, 10% CO_2, and N_2 was warmed and humidified by passage through two bubbler jars containing an aqueous solution of 20% $CuSO_4$ (to prevent mold growth) at 37°C and then fed directly into the chamber at a slow rate. The chamber was placed on a rocker table purchased from Bellco (Vineland, New Jersey) #6600. The speed of the rocker table was kept at 10-12 cycles per minute. The entire unit was housed in a National Appliances water jacketed incubator (#3212) and kept at a temperature of 37°C.

Once each 24 hours after the incubation was begun, the cultures were removed from the incubator and 0.1 ml of a nutrient mixture was added. The mixture which was used was that of Mosier, which consisted of 2x supplemented MEM and fetal bovine serum in a 1:1 ratio. A sterile disposable 1 ml syringe was used to introduce the

Plate 1. Incubation equipment.

Plate 2. Hemolytic plaque surrounding a spleen cell which is producing 19S antibodies against sheep red blood cells.


ROCKER PLATFORM
115 VOLTS A.C. 50-60 CYCLES
CAT. NO. 8000
Bellco Glass, Inc.
VINELAND, NEW JERSEY
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POWER
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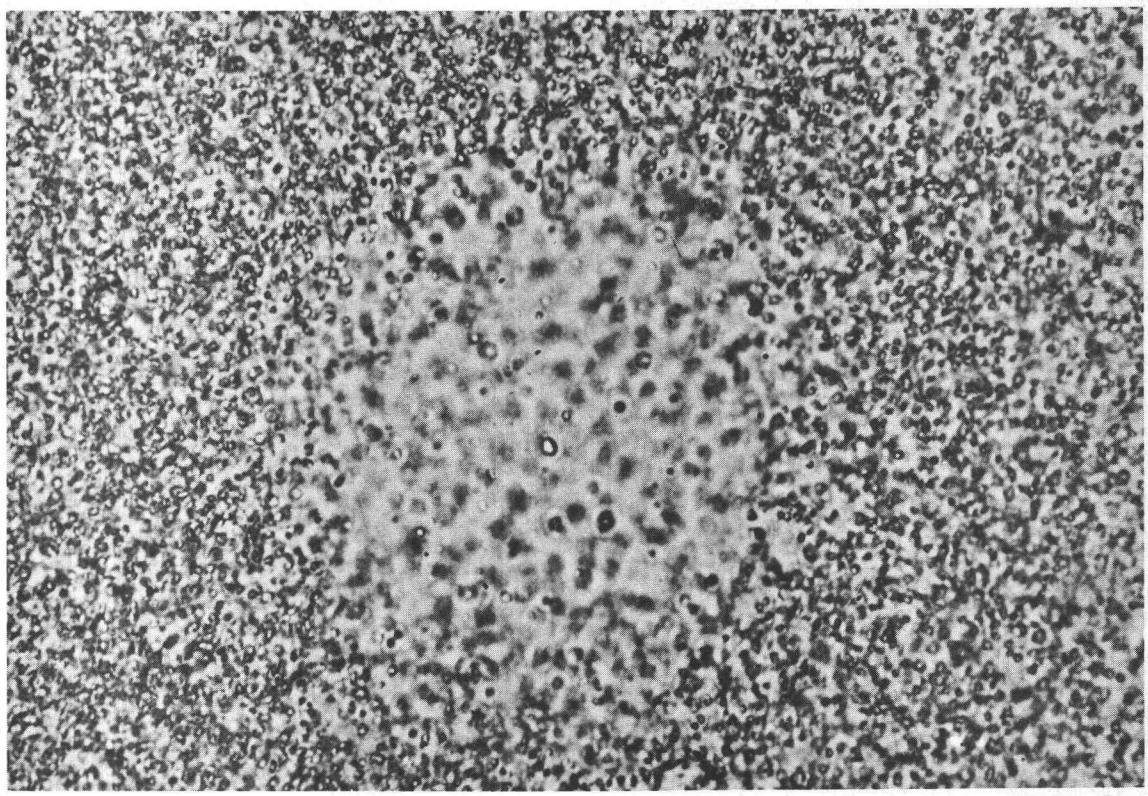


feeding mixture to the culture dishes. All work done upon open culture dishes was carried out under the protection of the sterile hood.

Assay for Plaque-Forming Cells

The assay for plaque-forming cells was a modification of that devised by Jerne et al. (38). Jerne detected cells producing antibodies against sheep erythrocytes by immobilizing spleen cells and sheep erythrocytes in an agar medium and then, after a period of incubation, introducing complement to the surface of the thin agar layer. During the incubation the antibodies produced diffuse into the medium and attach to the erythrocytes in the immediate vicinity of the antibody producing cell. Upon the addition of complement, each sheep erythrocyte to which 19S antibodies have attached lyses, releasing into the medium its cytoplasm containing hemoglobin. This is observed as a plaque, which is a small clear area on an otherwise uniform background of red blood cells.

The modification originated by Mishell and Dutton (48) consisted of the use of highly purified agarose in place of agar and DEAE cellulose, the use of microscope slides instead of petri dishes, and incubation in the presence of complement during the entire course of plaque development instead of developing the already-formed plaques in a second incubation. Microscope slides (27 x 75 mm) which were frosted on one end were used in this assay procedure.

In order to insure adherence of the agarose layer to the slide, each slide was dipped into a hot solution of 0.1% agarose in glass distilled water and then dried in the oven. The resulting clear thin layer of agarose that is deposited binds well to both the glass and another agarose gel layer that is added later.

The thickened tissue culture medium used for the detection of plaque-forming cells was prepared by the following method. Fifty mg of agarose was mixed with 9 ml of glass-distilled water and then dissolved either by autoclaving the mixture for ten or 15 minutes, or by heating the mixture in a boiling water bath. After the solution had cooled to below 60°C, one ml of 10x MEM without bicarbonate was added. The pH was adjusted to neutrality by the addition of 1N NaOH and then 0.35 ml portions of this mixture were pipetted into (10 x 75 mm) test tubes kept at 45°C in a water bath. These tubes of agarose were usually prepared at least an hour before the pouring of the assay slides.

For the determination of the numbers of plaque-forming cells in a culture, the cells were removed from the culture dishes by scraping with a rubber policeman and transferring the cells and media to a capped tube in an ice bath. One ml of MEM without bicarbonate was added to each dish and the process was repeated to dislodge the remaining cells. The washings were then recombined with the original culture fluid. Usually cells from several cultures

were pooled before the assay. The combined cells and washings were then centrifuged for ten minutes at 600 x g, the supernate was removed and the cells resuspended in MEM without bicarbonate. Aliquots of this suspension were used in the assay, for cell counts and for viability determinations.

At the time of assay 10 μl of a 33% suspension of sheep erythrocytes in either saline or tissue culture medium was added to each of the tubes of agarose, followed by 0.1 ml of spleen cells from the culture to be tested. The contents of the tube were quickly mixed and poured over the surface of an agarose-treated microscope slide, being careful to coat the entire non-frosted portion of the slide except for a 1/8 inch strip at the end. The agarose was allowed at least five minutes to set and then the slides were inverted and placed on the shallow plexiglas troughs. These troughs were then transferred to the 37°C incubator and the area beneath the slides was flooded with a solution of reconstituted guinea pig serum diluted 1:10 with freshly prepared Earle's balanced salt solution without bicarbonate. After incubation for one hour the slides were removed from the trays and the plaques (clear areas of lysed erythrocytes around 19S antibody-producing cells) were counted using a darkfield colony counter. The slides were held some distance from the actual front plate of the counter to obtain the best visual resolution of the plaques. Each plaque was marked with a dot of ink as it was counted to prevent

duplication in the counting of individual plaques.

If the plaques to be determined were produced by 7S antibodies, then the slides were incubated in a dilute solution of rabbit anti-(mouse gamma G-globulin) serum. After this incubation the liquid is poured away and the slides are flooded with a solution of complement to develop the 19S and 7S plaques. The second incubation and counting are identical to the methods for 19S plaques.

From time to time plaques produced by this agarose slide method were observed under the microscope and usually a single large cell could be found in the center of the plaque. An example of a 19S hemolytic plaque is shown in Plate 2. As can be seen in the photomicrograph, it is sometimes difficult to identify the plaque-forming cell due to the thickness of the agarose layer and the often high flux of spleen cells. No morphological identification of the cell types producing antibodies was attempted in this work.

Determinations of Cell Numbers

A hemocytometer was used for all cell counts. In order to determine the total number of nucleated cells, staining was accomplished with crystal violet.

Viability determinations were based on the ability of viable cells to exclude the dye trypan blue (6). The trypan blue was prepared as a stock solution of 0.2% in distilled water. Immediately before use

four parts of the dye stock solution was mixed with one part of a stock solution of 4.25% NaCl to render it isotonic. One part of this isotonic dye solution was added to three parts of a diluted cell suspension and after five minutes the number of stained cells was determined. The viable cells were represented by the number of total nucleated cells as determined by the crystal violet staining, less the number of cells stained by trypan blue.

EXPERIMENTAL AND RESULTS

The purpose of this research project is to attempt further definition of the biochemical processes involved in the induction of an immune response. Studies by several workers of actinomycin D effects on the immune response suggested that messenger RNA synthesis is necessary for antibody synthesis (60, 61, 64) and that the messenger RNA responsible for antibody synthesis is a relatively long-lived species (1, 26, 43). By establishing the temporal relationship between messenger RNA inhibition and the inhibition of the immune response, it should be possible to define the time during the induction when all messenger RNA species necessary for antibody synthesis are present.

In Vitro Immune Response

As described in the introduction the in vitro induction of the immune response offers the obvious advantages of better control of the time of induction and the amounts of antigen and actinomycin D which are present with a given amount of tissue, as well as the possibility of carrying out several determinations on the tissues from one animal or group of animals. The method of inducing the immune responses used in this work was developed by Mishell and Dutton and modified by Mosier (50) with respect to the nutrients added

during culture. The immune response seen under such experimental conditions was shown by these authors to produce an exponential rise in the number of hemolytic-plaque-forming cells beginning 48 hours following addition of the antigen and continuing for two or three days (48). This is quite similar to the kinetics of the in vivo immune response in mouse spleen. Figure 2 shows a typical immune response induced using this system (as described in the methods section). For most of the work presented here, however, only the later stages of the response were observed due to limitations in the numbers of cultures available for one experiment and the relatively high numbers of cells which must be assayed during the early stages of the response.

Actinomycin D Effects on RNA and Protein Synthesis

In order to prove useful in this type of experiment a concentration of actinomycin D should be used which will bring about some inhibition of RNA synthesis with hopefully little or no adverse effect on the synthesis of needed cellular proteins. The effects of actinomycin D upon RNA and protein synthesis in spleen cells under culture conditions identical to those for the induction of the immune response are shown in Table 2. The spleen cells were cultured in the presence of ^{14}C-labeled proline or uridine. At the end of the indicated incubation period the cells were collected, several volumes of distilled

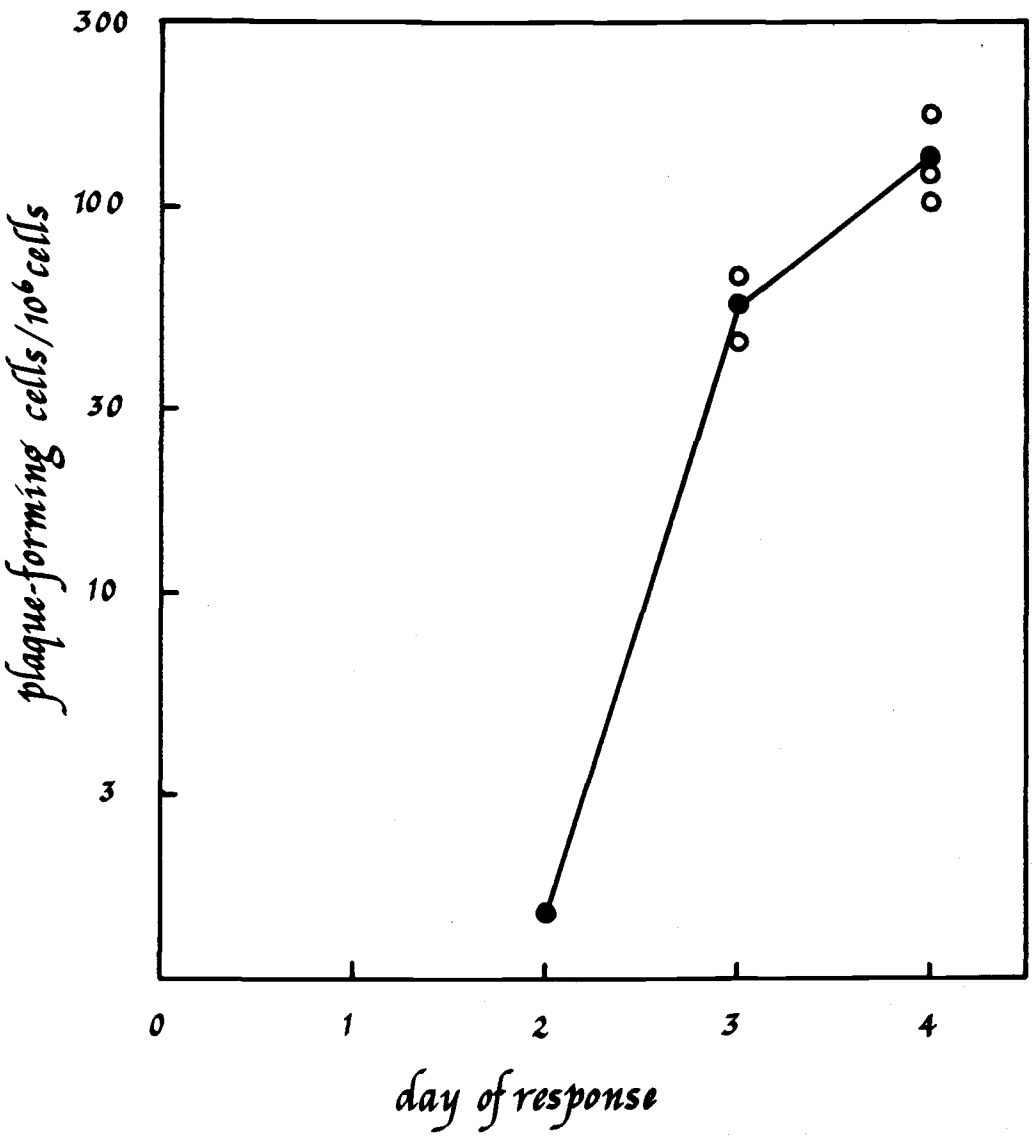


Figure 2. Typical in vitro immune response.

Table 2. Effect of actinomycin D on RNA and protein synthesis in the culture system.

Exp. #	Labeled substrate	Period of incubation	CPM control	CPM act. D	Conc. act. D	Inhibition %
1	^{14}C-Uridine	13 hr	1173 1529 1510 Av (1404)	146 104 246 Av (165.3)	1.25 μg/ml	88.2%
1	^{14}C-Proline	13 hr	314 473 Av (393.5)	166 182 Av (174)	1.25 μg/ml	55.7%
2	^{14}C-Proline	10 hr	809	494 581 Av (537)	1.25 μg/ml	34%
2	^{14}C-Proline	10 hr	809	973 980 Av (976)	0.125 μg/ml	(20% enhancement)
3	^{14}C-Proline	17 hr	603 603 605 Av (604)	392 403 Av (397.5)	1.25 μg/ml	33%

water were added to lyse the cells, and an equal volume of 50% cold trichloroacetic acid was added to each of the lysed cell suspensions. The precipitates were collected on membrane filters and washed repeatedly with 10% cold trichloroacetic acid. The filters were then dried and counted in a Packard Tri Carb liquid scintillation counter using a toluene based scintillation fluid. (In two of the experiements tritium labeled uridine was used, but the quenching was so great using this method that the data were not useful, and therefore for two of the experiments only the ^{14}C-proline incorporation data are given.)

It can be seen from Table 1 that in this rough estimate of the effect of actinomycin D on the cell's synthetic processes a concentration of 1.25 μg actinomycin D per ml of culture fluid gave nearly 90% inhibition of RNA synthesis while inhibiting only one third to one half of the total protein synthesis in the cultures. The 0.125 μg/ml concentration of actinomycin D gave no inhibition of protein synthesis, and in fact gave a 20% enhancement. It was therefore decided to use actinomycin D concentrations between or lower than these two values for the inhibition of plaque-forming-cells, since protein synthesis would be relatively unaffected and RNA synthesis partially blocked.

Actinomycin D Effects on Primary and Secondary Immune Responses

Several experiments were carried out on both primary and

secondary in vitro immune responses to determine the effect of the presence of actinomycin D concentrations of 0.1 to 0.125 μg/ml throughout the entire culture period. The results of these experiments are summarized in Table 3.

It can be seen that this low concentration of actinomycin D is sufficiently high to give nearly complete inhibition of the immune responses. In the one case where the number of plaque-forming cells was determined on both day three and day four of the response the actinomycin D effect was not quite as severe when measured on day three, even though the PFC numbers developed at that time were lower.

Since the 0.1 to 0.125 μg/ml concentration range of actinomycin D did completely inhibit the development of plaque-forming cells directed against the antigen, and incidently also blocked the development of plaque-forming cells due to the presence of cross-reacting antigens in the fetal bovine serum of the culture media, this concentration was used to investigate the effect of actinomycin D on the later stages of the in vitro immune induction. Table 4 contains the results of experiments utilizing both primary and secondary responses in which the drug was added after various stages of the immune induction had been reached. The presence of actinomycin D in the assay media does not significantly reduce the numbers of plaque-forming cells, as can be seen in the 96 hour value from experiment-B.

Table 3. Effect of actinomycin D added initially upon the PFC induction process.

Exp #	Actinomycin D (μg/culture)	Plaque forming cells*		Inhibition %
		Control	Actinomycin D	
Primary				
p-18	0.125	$477/10^6$ tot	$2.9/10^6$ tot	99.4%
p-19	0.1	2360/culture	9.2/culture	99.6%
Secondary				
s-5	0.125	$42.5/10^6$ tot	$0.3/10^6$ tot	99.4%
s-6	0.125	$14.5/10^6$ tot	$0.1/10^6$ tot	99.3%
s-8	0.125	$132/10^6$ tot	$0.8/10^6$ tot	99.4% (day 4)
s-8	0.125	$56/10^6$ tot	$2.7/10^6$ tot	95.2% (day 3)
s-10	0.1	1237/culture	3.3/culture	99.7%

*(This data is usually reported on a basis of PFC/total nucleated cells or PFC/culture basis instead of PFC/viable cells because the viability test used in this work is not accurate for the low viability ranges found in the actinomycin D-treated cultures, and even the experimental cultures are often only 20% viable by this test at the time of assay.)

Table 4. Effect of actinomycin D added to cultures at later stages of the immune responses.

Type of response	Exp. #	Actinomycin D concentration	Time added	Day assayed	PFC/10^6 total cells	% Inhibition
Secondary	A	--	--	4	(585-667)	--
	A	--	--	4	772	--
	A	-- (Control average)	--	4	595 (655)	--
	A	0.1 μg/ml	24 hr	4	2.3	99.65%
	A	0.1 μg/ml	48 hr	4	12.3	98.1%
	A	0.1 μg/ml	72 hr	4	10.7	98.3%
	A	1.0 μg/ml	24 hr	4	2.1	99.7%
	A	1.0 μg/ml	48 hr	4	4.5	99.3%
	A	1.0 μg/ml	72 hr	4	10	98.5%
Secondary	B	--	--	4	132	control
	B	0.125 μg/ml	0 hr	4	0.8	99.4%
	B	0.125 μg/ml	24 hr	4	3.65	97.2%
	B	0.125 μg/ml	48 hr	4	5.25	96.0%
	B	0.125 μg/ml	72 hr	4	3.25	97.5%
	B	0.125 μg/ml	96 hr	4	120	9%
	C	--	--	3	40 ± 22	control
	C	0.1 g/ml	48 hr	3	1.75	95.6%
Primary	C	--	--	4	74 ± 26	control
	C	0.1 μg/ml	72 hr	4	13.7 ± 4	81%

However the presence of actinomycin D in the cultures for any period of 24 hours or more at any stage of the induction was sufficient to cause nearly complete inhibition of the response. This type of inhibition cannot be simply due to blockage of cell division because with the 72 hour additions a sizeable number of plaque-forming cells were present in the cultures at the time of drug addition and these two were lost as a result of that treatment. The results of experiment A show that there are similar inhibitory effects when 0.1 μg/ml or 1.0 μg/ml concentrations of actinomycin D are used.

Sensitivity of Cultures to Initial Additions of Actinomycin D

Experiments were undertaken to determine the sensitivity of the immune induction to actinomycin D. In order to accomplish this serial dilutions of actinomycin D were prepared throughout the concentration range from 1 μg/ml to 10^{-3} μg/ml and added to the spleen cell cultures at the same time as antigen. Three cultures were used for each concentration and the cells from each group of three cultures were combined at the time of assay. Since the relative magnitude of the response compared with the control cultures is needed for this determination rather than the absolute magnitude of the response, the results are expressed in terms of plaque forming cells recovered per culture. The sensitivity data for both primary

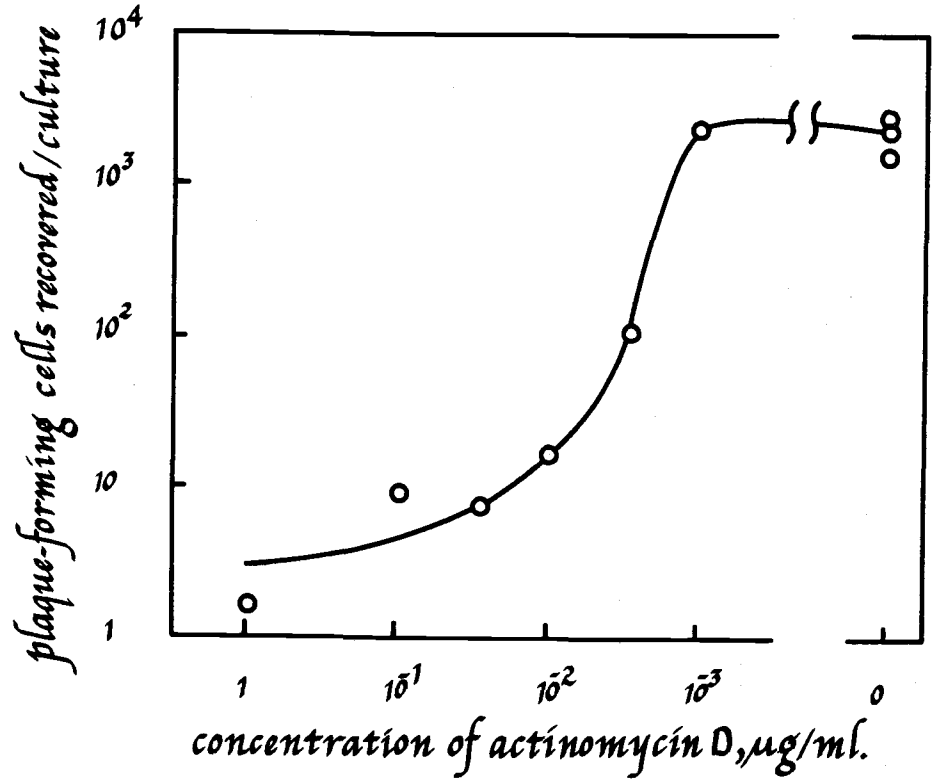
and secondary responses is contained in Table 5. The results for each determination are shown graphically in Figures 3 (secondary) and 4 (primary).

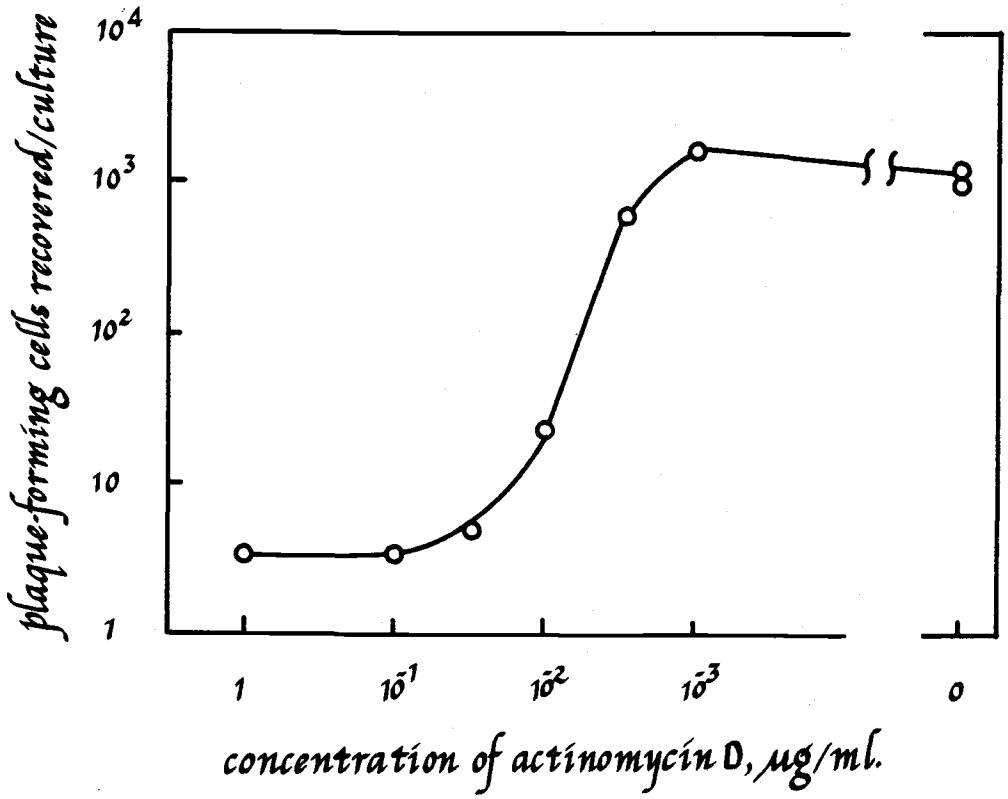
Table 5. Sensitivity of primary and secondary in vitro responses to actinomycin D added initially.

Actinomycin D concentration	Primary response		Secondary response	
	# of cultures	PFC/culture	# of cultures	PFC/culture
1 μg/ml	3	1.67	3	3.3
10^{-1} μg/ml	3	9.2	3	3.3
3×10^{-2} μg/ml	3	7.5	3	5
10^{-2} μg/ml	3	16.7	3	23
3×10^{-3} μg/ml	3	108	3	618
10^{-3} μg/ml	3	2307	3	1653
0 μg/ml	1	2360	2	950
0 μg/ml	1	2760	2	1237
0 μg/ml	1	1550		
	average	(2223)	average	(1094)

Figure 3. Sensitivity of secondary in vitro response to actinomycin D added initially.

Figure 4. Sensitivity of primary in vitro response to actinomycin D added initially.


plaque-forming cells recovered/culture
10^4
10^3
10^2
10
1
1
10^{-1}
10^{-2}
10^{-3}
0
concentration of actinomycin D, μg/ml.


plaque-forming cells recovered/culture
10^4
10^3
10^2
10
1
1
10^{-1}
10^{-2}
10^{-3}
0
concentration of actinomycin D, μg/ml.

Actinomycin D Effect on Separate Cell Populations

Some of the early stages of the immune induction, i.e. the interaction of macrophages with the antigen, are essentially complete after one hour (50). Since actinomycin D sensitivity seems to be greatest if the drug is present throughout all of the induction processes, it is important to determine the effect of the drug when present only during the early stages. In order to carry out this type of experiment, the two cell population culture system of Mosier was used. Mosier showed that the spleen population could be resolved into two cell types on the basis of the cell's ability to adhere to the surface of the culture dish during a short incubation period. The resulting "lymphocyte-rich" and "macrophage-rich" fractions were shown by him in the case of the primary response to be incapable of mounting an immune response. However, when the two populations were reunited in roughly the same proportions as in the original cell suspension, an immune response occurred which was equivalent to that of the original cell suspension.

Since the small lymphocyte has been implicated as the carrier of long-term immunological memory (29), it was feared that the secondary response may prove less than useful in this work because the lymphocyte-rich fraction might thus be capable of mounting an immune response in the absence of the macrophage-rich fraction.

To test this hypothesis Mosier's work was duplicated with a spleen cell suspension from mice which had already undergone a primary immune response to the SRBC antigen. The results from this work are shown in Table 6. Since the lymphocyte-rich fraction in this case was in fact able to support an immune response, it was decided to study only the primary response for the determination of the effect of actinomycin D on the early induction events.

Table 6. The secondary immune response in lymphocyte-rich and macrophage-rich fractions of the spleen cell suspension.

Secondary	PFC/10^6 tot	PFC/10^6 viable
Normal cell population	26.3 ± 7.4	53.1 ± 15
Reconstituted	99.4 ± 40	147 ± 58.6 (SRBC only exposed to MR-) (fraction for first hour)
Lymphocyte-rich + SRBC	37.1 ± 29.4	99 ± 78
Macrophage-rich + SRBC	0	0

Mosier (50) showed that it is only necessary that the macrophage-rich population of spleen cells be exposed to the sheep erythrocyte antigen for one hour in order for a normal in vitro immune response to occur after recombination with the lymphocyte-rich cell population. Table 7 shows the results of an experiment of this type and the effects of actinomycin D treatment of the macrophage-rich

cell population.

The response of the recombined cell fractions is somewhat lower than the control value for the original cell suspension, but this may only indicate a loss of some of the macrophages during the process of rinsing away the excess antigen before recombining the cell fractions. When the incubation of macrophages and antigen was carried out under normal conditions and actinomycin D (0.1 μg/ml) was added only after the two cell populations were recombined, the response was limited to 1.7% of the control value. This is in accord with the results of later additions of actinomycin D on the normal culture system. If actinomycin D was present only for the first hour when the antigen interaction takes place, the response was inhibited to levels of 4.6% and 1.3% of the control value by 0.1 μg/ml and 1.0 μg/ml drug concentrations respectively. Thus the response is still inhibited though the entire lymphocyte-rich population remains untreated by the drug. In an attempt to separate the effects of the drug on uptake of the antigen from later stages of the response, macrophage-rich populations were treated with the drug and then antigen was added after the two cell populations had been recombined. The response in this case was virtually identical to that obtained when the antigen was present only during the one hour period of drug treatment. The effect of actinomycin D treatment thus seems to be one of virtual removal of a large portion of the needed

macrophages, either by causing long lasting damage to the macrophages of by lowering the actual population of macrophages by causing the cells to detach from the culture dish surface.

This second possibility was checked by determining the macrophage population before and after actinomycin D treatment. Macrophage-rich (adherent) cell populations were prepared from a spleen cell population and then incubated for one hour with either normal culture media or media containing 0.1 μg/ml actinomycin D. Following the incubation, the culture fluids were removed and the cells which became detached were counted. The culture dishes were also rinsed as usual and then culture media containing EDTA (30 mm) was added for a second incubation to cause the macrophages to become detached from the dishes. These culture fluids were also examined for detached cells. The results of this work are shown in Table 8.

Although sizeable numbers of cells did detach from the culture dish surface, and in fact only about one third remain attached at the end of the treatment period, the same pattern is seen in the control cultures as in the actinomycin D-treated cultures. Presumably the actinomycin D inhibition seen in the case of the one hour initial drug treatment is due to some long lasting effect on the cells of the macrophage population.

Table 7. Effect of actinomycin D on macrophage-rich population in immune induction.

	PFC/10^6	Sheep RBC		Actinomycin D	
		1st hr	later	1st hr* (μg/ml)	later (μg/ml)
Normal - control	477	yes	yes	--	--
Recombined - control	225	yes	no	--	--
Recombined - act. D	3.9	yes	no	--	0.1
Recombined - act. D	10.4	yes	no	0.1	--
Recombined - act. D	2.9	yes	no	1.0	--
Recombined - act. D	10.7	no	yes	0.1	--

*For all data except the normal control, the first hour treatment with either antigen or actinomycin D involves only the macrophage-rich fraction, whereas later treatment affects both macrophage-rich and lymphocyte-rich fractions.

Table 8. Effect of actinomycin D on macrophages adhering to the surface of culture dish.

		Total nucleated cells/culture	# of cultures
Original cell suspension		1.76×10^7 (1.35×10^7 viable)	
Macrophage-rich population		$8.4 \times 10^5 \pm 2.1 \times 10^5$	5
Supernatant after 1 hr incubation			
Control		$3.5 \times 10^5 \pm 1.3 \times 10^5$	6
Actinomycin D	1.0 μg/ml	$2.7 \times 10^5 \pm 1.1 \times 10^5$	5
Actinomycin D	0.1 μg/ml	$3.4 \times 10^5 \pm 1.0 \times 10^5$	5
Adherent cells after 1 hr incubation			
Control		$2.6 \times 10^5 \pm 0.9 \times 10^5$	7
Actinomycin D	1.0 μg/ml	$2.8 \times 10^5 \pm 1.1 \times 10^5$	6
Actinomycin D	0.1 μg/ml	$2.4 \times 10^5 \pm 0.6 \times 10^5$	6

In Vitro Effects of Actinomycin D on an Immune Response Initiated In Vivo

Some recent work reported by Gilmore Harris (33) with respect to the effects of actinomycin D on an immune response begun in vivo and continued in vitro has cast doubts on many of the conclusions that might be reached from this and other experiments utilizing actinomycin D to inhibit the immune response. In Harris' experiments the immune response was studied with rabbit spleen tissues from animals which had already been immunized and had received a booster injection of antigen two to three days before the spleens were excised. Short term effects of actinomycin D were the inhibition of RNA synthesis and a drop in the number of plaque forming cells. In longer term studies, however, puromycin and actinomycin D in a wide range of concentrations stimulated cells which had been incubated in the presence of antigen for three or four days into production of antibodies in much higher numbers than were present in the control cultures which were not treated with the drug. In fact the plaque forming cell numbers in the actinomycin D-treated cultures were higher than the number of plaque-forming cells in the original cell suspension, which was the highest value in the control series.

In order to determine if the same type of stimulatory effects were possible using the mouse immune system in vitro this same schedule of immunization and drug additions to the cultures was

followed using mouse spleen tissues and the culture conditions described in the methods section. An actinomycin D concentration of 1.25 μg/culture was added to cultures on each of five days and the number of plaque-forming cells was determined 24 hours later. The results from this study are shown in Table 9. As the results indicate the same stimulatory effects are not in evidence, and any increases in the drug treated cultures seem to be correlated with increases in the number of plaque-forming cells in the control series of cultures prior to the period of drug treatment. A comparison of this data and that reported by Harris will be found in the discussion section.

Table 9. Effect of actinomycin D on secondary PFC response in vitro which was initiated in vivo.

CULTURES		PFC/10^6 spleen cells (days)						
		0	1	2	3	4	5	5 (7S)
Controls (C)		89	---*	---*	404	86	45	(500)
SRBC		--	173	---*	4400	2083	1200	(8800)
Act. D on day 0	C		8.8					
	SRBC		7.6					
Act. D on day 1	C			7.8				
	SRBC			14.0				
Act. D on day 2	C				10			
	SRBC				47			
Act. D on day 3	C					37		
	SRBC					199		
Act. D on day 4	C						46.5	
	SRBC						83.5	

*The missing values for day 1 and 2 are due to the fact that the assay slides were unreadable due to the high concentration of hemolysin in the media and the resultant lysing of all SRBC's on the assay slide. For later determinations the cell suspensions were always centrifuged and resuspended in fresh media.

DISCUSSION

Actinomycin D Effects on the In Vitro Induction of Immune Responses

Figures 5 and 6 summarize for the primary and secondary responses respectively the pertinent data relating the plaque-forming cell responses to different concentrations of actinomycin D at various stages of the response. Also included in Figure 5 is the 24 hour sensitivity data reported by Uyeki and Llacer (70), who also used the culture system of Mishell and Dutton. The level of inhibition was great even on the later stages of the immune responses when the drug concentrations of 0.1 μg/ml or greater were used. Because of this very low level of response in the drug treated cultures it is difficult to draw conclusions as to a stage in the response after which the drug has a much reduced effect except to point out that the drug inhibition is somewhat less severe by the end of 24 hours. However from a comparison of the drug effects at lower concentrations which were determined for the initial stages of the response with the 24 hour values reported by Uyeki and Llacer this change in sensitivity is very distinct. For instance the dose of actinomycin D (0.01 μg/ml), which caused greater than 99% inhibition of the response when present during all stages of the response, inhibits the same system by less than 50% if added after the first 24 hours of culture. Therefore

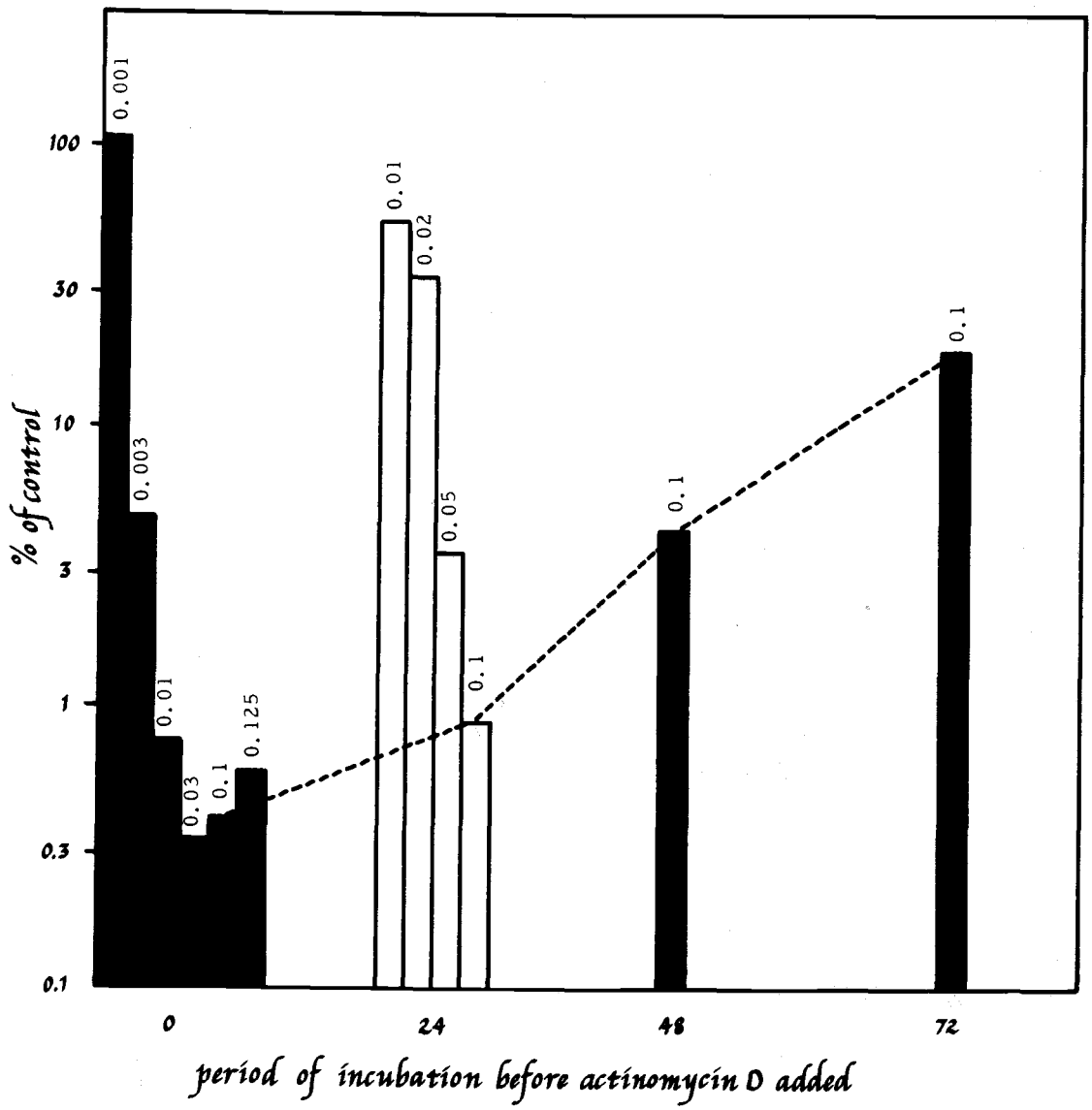


Figure 5. Sensitivity of primary PFC response to actinomycin D added at various stages of the response. Actinomycin D concentrations in μg/ml are shown above each bar.

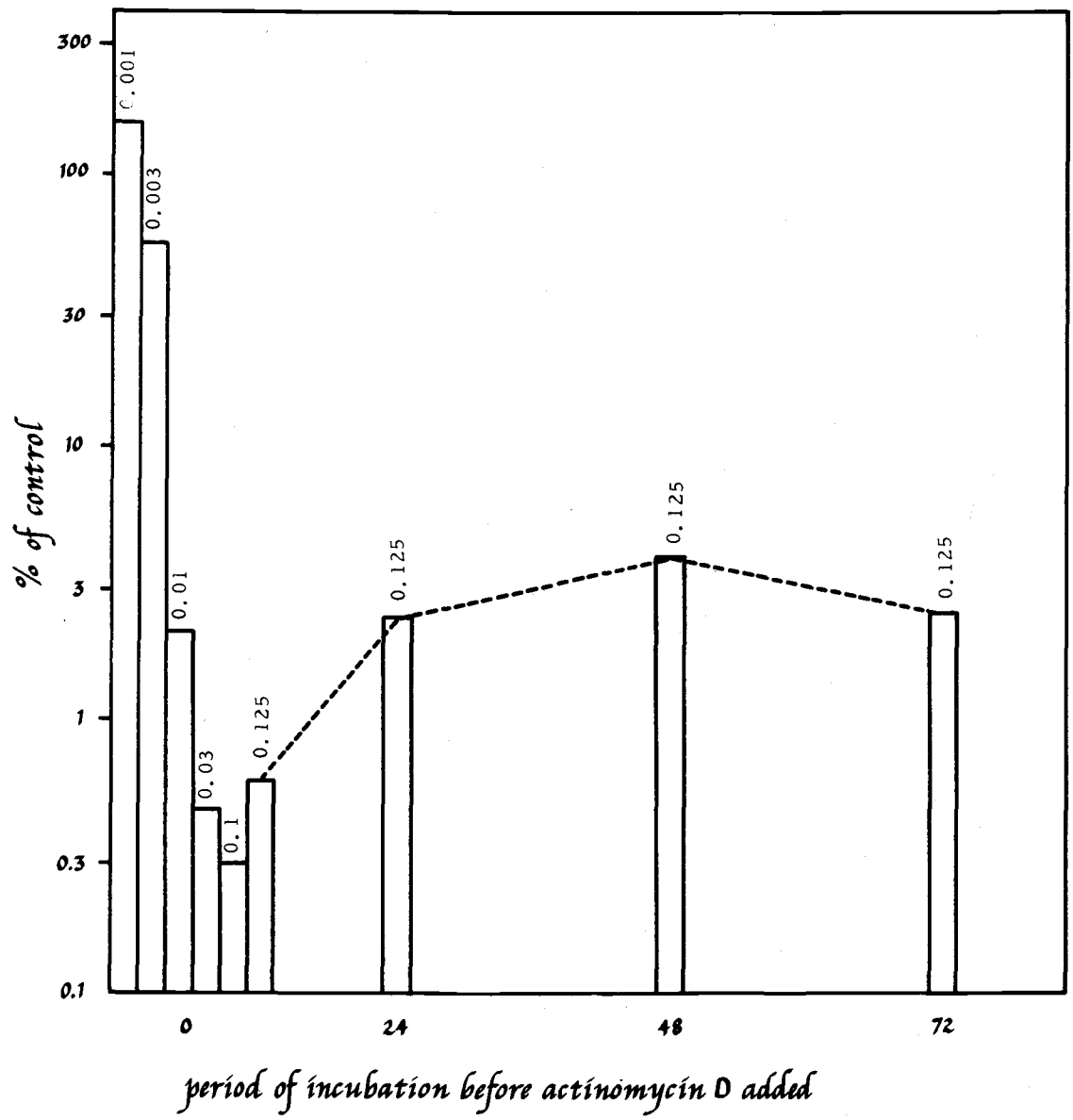


Figure 6. Sensitivity of secondary PFC response to actinomycin D added at various stages of the response. Actinomycin D concentrations in μg/ml are shown above each bar.

it is evident that at least one event in the primary immune induction is inhibited by very low concentrations of actinomycin D and is completed to a large extent by the end of the first day of the immune induction. Ultimately, if the sensitivity of the culture system to very low levels of actinomycin D is determined at stages even earlier than 24 hours, a better definition of the RNA synthesis patterns of the early induction processes would result.

In this consideration of the early events of the immune response, it is important to note that a one hour treatment of the macrophage population with actinomycin D (0.1 μg/ml) causes extensive suppression of the immune response, even if antigen is present after the removal of the drug. This may be due to selective killing of cells important to the immune induction, as will be discussed in a later section, or enough actinomycin D may remain bound to the DNA of the cell to prevent the occurrence of the early, highly actinomycin D sensitive step in the induction process.

Aside from the recent brief report by Uyeki and Llacer mentioned above, all of the actinomycin D sensitivity work utilizing the in vitro initiation of primary immune responses has come from the laboratory of Alain Bussard. Bussard's work involved the primary response by mouse peritoneal cells incubated in a gum of carboxymethylcellulose containing sheep erythrocytes as the antigen. In the first report (10) the gum containing the cells was mounted between a

slide and coverslip for the entire incubation period. For this reason only the effects of initial drug addition could be ascertained. Actinomycin D, at a concentration of 2 μg/ml, completely prevented the primary response. Furthermore, by using cells from an immunized animal it was shown that the actinomycin did not prevent development of plaques around cells already synthesizing antibodies although the numbers of such cells was depressed somewhat. In this culture system the kinetics of PFC development could be observed continuously throughout the culture period. A lag period of 18-20 hours duration was observed, followed by a logarythmic increase in the numbers of plaque-forming cells. After 48 to 72 hours the numbers of plaque-forming cells became stabilized, probably due to the decay by this time of most of the complement in the system. Although this in vitro culture system seems to be the ideal system for the study of the early events in the primary immune response, I could never reproduce Bussard's results using several mouse strains, including C3H, which was reported to give the best responses.

Bussard and Lurie (12) developed a two-step culture system, in which the peritoneal cells were first incubated for 24 to 48 hours with antigen in either liquid media or the CMC gum in siliconized glass tubes, and then the slides were prepared. When actinomycin D (1 μg/ml) was present in the system for the first 18-26 hours of incubation in liquid media, no plaque-forming cells developed. When

When the same concentration of actinomycin D was added after 24 to 48 hours of normal incubation, the number of antibody plaques was depressed but not completely. Although it is difficult to make direct comparisons from Bussard's system to that used in this work because of differences in the cell types and the course of the normal response, both systems agree in the finding that at early stages the immune responses can be completely blocked and that at later stages the actinomycin D inhibits the response, but not completely. Bussard's system suffers from the drawback that the preincubation in gum or liquid media needed for periodic drug additions always caused a great reduction in the number of plaque-forming cells as compared to the cultures placed on a slide from the beginning of the induction.

With respect to the secondary immune response initiated in vitro, no other results utilizing a comparable system are available for comparison. However some studies on other types of secondary in vitro immune responses have been reported. Uhr in 1963 (68) utilized a system developed by Michaelides and Coons (46) for inducing a secondary response to T_2 phage in cultures of lymph nodes taken from rabbits six to 34 weeks after the primary immunization. Uhr reported the continued maintenance of a low level of T_2 inactivating antibodies in non-stimulated cultures. This continued antibody synthesis, which represents the later stages of the primary response, was inhibited during the 15 days of culture by actinomycin D in

concentrations as low as 5×10^{-8} M (6.3×10^{-2} μg/ml). If T_2 antigen was added to the organ cultures, a secondary response is initiated resulting in a rise in the amount of anti-T_2 antibodies produced. It was found that 5×10^{-8} M actinomycin D virtually eliminated this response also.

Ambrose in 1966 (1) used the same culture system except that the antigens used were bovine serum albumin and diphtheria toxin. The total amount of antibody that was synthesized during the 18 day culture period was correlated with the various concentrations of actinomycin D continuously present in the culture medium. The data from this work is plotted on Figure 7 and Table 10 for comparison with the inhibition of data of Uyeki and Llacer and from this thesis. Note that there is a relative correspondence between the two types of secondary experiments at the highly inhibitory amounts, even though in one case the amount of antibody produced is being measured and in the other case the number of cells producing antibody is reported. The divergence of the two curves at the lower concentrations is expected, since even though a cell's total antibody production may be smaller than normal in the presence actinomycin D it may be a sufficient amount to allow its identification as a plaque forming cell. Ambrose also found that if the cultures were allowed to progress normally for the first nine days following antigen introduction, followed by addition of actinomycin D (0.02 μg/ml), antibody synthesis

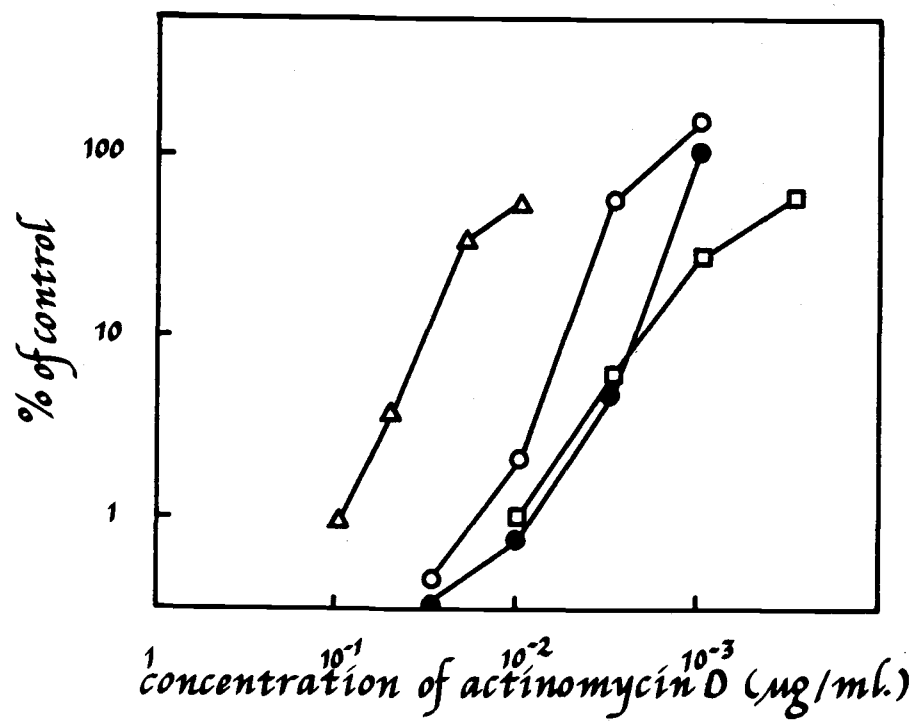


Figure 7. Comparison of actinomycin D effects on immune induction.

Table 10. Comparison of actinomycin D effects on immune induction.

Source	Type of response	Actinomycin D concentration	Value (PFC)	% of control
Ambrose	Secondary	0		100%
□—□		0.01 µg/ml		1%
		0.003 µg/ml		6%
		0.001 µg/ml		27%
		0.0003 µg/ml		58%
Uyeki	Primary	0	111	100%
△—△		0.1 µg/ml	1	0.9%
		0.05 µg/ml	4	3.6%
		0.02 µg/ml	38	34%
		0.01 µg/ml	60	54%
Thompson	Primary	0	2223	100%
●—●		0.03 µg/ml	7.5	0.34%
		0.01 µg/ml	16.7	0.75%
		0.003 µg/ml	108	4.85%
		0.001 µg/ml	2307	104%
Thompson	Secondary	0	1094	100%
○—○		0.03 µg/ml	5	0.46%
		0.01 µg/ml	23	2.1%
		0.003 µg/ml	618	56.5%
		0.001 µg/ml	1653	151%

continued normally for three or four days and then came to an abrupt halt. This work shows again that the induction phase of the response is much more sensitive to the actions of metabolic inhibitors than is the production of antibodies itself. This work would also indicate that the messenger RNA for actual synthesis of antibodies is synthesized during this period, or else it is stable for at least the three or four day period to allow for continued synthesis of antibody.

Actinomycin D Effects on *In Vitro* Continuation of Immune Responses Initiated *In Vivo*

Many studies of the effects of actinomycin D on the immune response in tissue culture have been done in which an advanced secondary response was initiated in the animal and then the tissues were transferred to a culture environment for the drug treatment. This method has the advantage of a high number of antibody-producing cells in the population to be studied, but has the disadvantage for comparison with this thesis in that all induction events happen before the study is initiated. Much of this work has recently been reviewed in an article by Tannenberg and Schwartz (67, p. 163-179) and so only those works which are useful in interpreting the results of this thesis will be included here. In interpreting these results it should be noted that the culture conditions for many of the short-term studies are not optimal for long-term cell viability, and this fact may

influence the results.

Lazda and Starr in 1964 (43) demonstrated that 1 μg/ml of actinomycin D inhibited RNA synthesis in cultured rabbit spleen cells from immunized rabbits but did not cause any appreciable depression of incorporation of labeled amino acids into specific antibody, nor depression of the hemagglutinin titer during the first 24 hours of culture. During the period of culture from 24 to 48 hours a depression of both RNA and antibody synthesis and also the hemagglutinin titer occurred. From this work it was concluded that the messenger RNA specific for antibody production had a rather long half-life.

Svehag in 1964 (65) found, however, that 1-10 μg/ml of actinomycin D interrupted the synthesis of anti-poliovirus-antibodies by spleen cells in vitro if treated for 30 minutes or longer. Actinomycin D treatment for only five to 15 minutes brought about partial suppression and antibody synthesis was slowly renewed upon the removal of the drug.

Strander in 1966 (64) studied the number of plaque-forming cells in short term (seven hour) cultures following secondary immunization. Actinomycin D in a concentration of 1 μg/ml had little or no effect for the first six hours of culture, but larger doses did cause decreases in PFC numbers. The highest dose (25 μg/ml) of actinomycin D caused the complete loss of plaque-forming ability in the population of cells after only 4.5 hours. Strander also demonstrated

that at high concentrations of actinomycin D (20 μg/ml) the drug needed to be present only during the first hour of culture to cause the PFC number to drop with further incubation, even if the drug is removed from the culture medium.

Stavitsky and Gusdon (61) prepared organ cultures of spleen and lymph node fragments from rabbits three days after a secondary injection of a soluble of a soluble antigenic protein. The effects of actinomycin D were noted upon the incorporation levels of ^{14}C-labeled uridine, Thymidine and Glycine into the RNA, DNA, non-antibody protein and specific antibody. Actinomycin D (1.0 μg/ml) brought about the cessation of incorporation of label into extracellular antibody after three hours, while 0.1 and 0.5 μg/ml doses allowed continued increase during the seven hour observation period. Actinomycin D (1.0 μg/ml) brought about a significant increase in the incorporation of label into total antibody for the first five hours even though RNA synthesis was inhibited. In another experiment 1 μg/ml actinomycin D stimulated antibody synthesis for the first seven hours, but caused about 40% inhicition at 24 hours.

Smiley, Heard and Ziff (60) studied the effect of actinomycin D and several other potent inhibitors of nucleic acid and protein synthesis on RNA and antibody formation by anamnestic rabbit lymphoid cells. The effects were observed for six hours only. Actinomycin D (1 μg/ml) gave 90% inhibition of RNA synthesis but no inhibition of

antibody synthesis. At doses in excess of 1 μg/ml increasing concentrations of actinomycin D produced a progressive decrease in synthesis of antibodies directed against bovine serum albumin. Figure 8 shows the data from two of these experiments on inhibition of RNA and protein synthesis as well as the data from this thesis on inhibition of the primary and secondary immune inductions, both plotted as a function of actinomycin D concentration. The purpose of this figure is to illustrate the much greater sensitivity of the induction process than one would expect from the RNA and protein inhibition studies. A possible explanation for this observation will be discussed later.

Gilmore Harris (33), who used a similar experimental system of spleen cell culture of tissue taken from an immunized rabbit two or three days following the secondary injection of sheep erythrocyte antigen was able to corroborate the results of several other workers concerning short term inhibitory effects of actinomycin D on the cultures. However he has also shown that, in cultures to which additional antigen has been introduced directly, actinomycin D in large amounts can stimulate large increases in the number of antibody-producing cells in the culture. This result calls into serious question the interpretation of actinomycin D effects in the in vitro immune responses and possibly other experiments as well.

Using this culture system, Harris showed that 6.7 μg/ml of

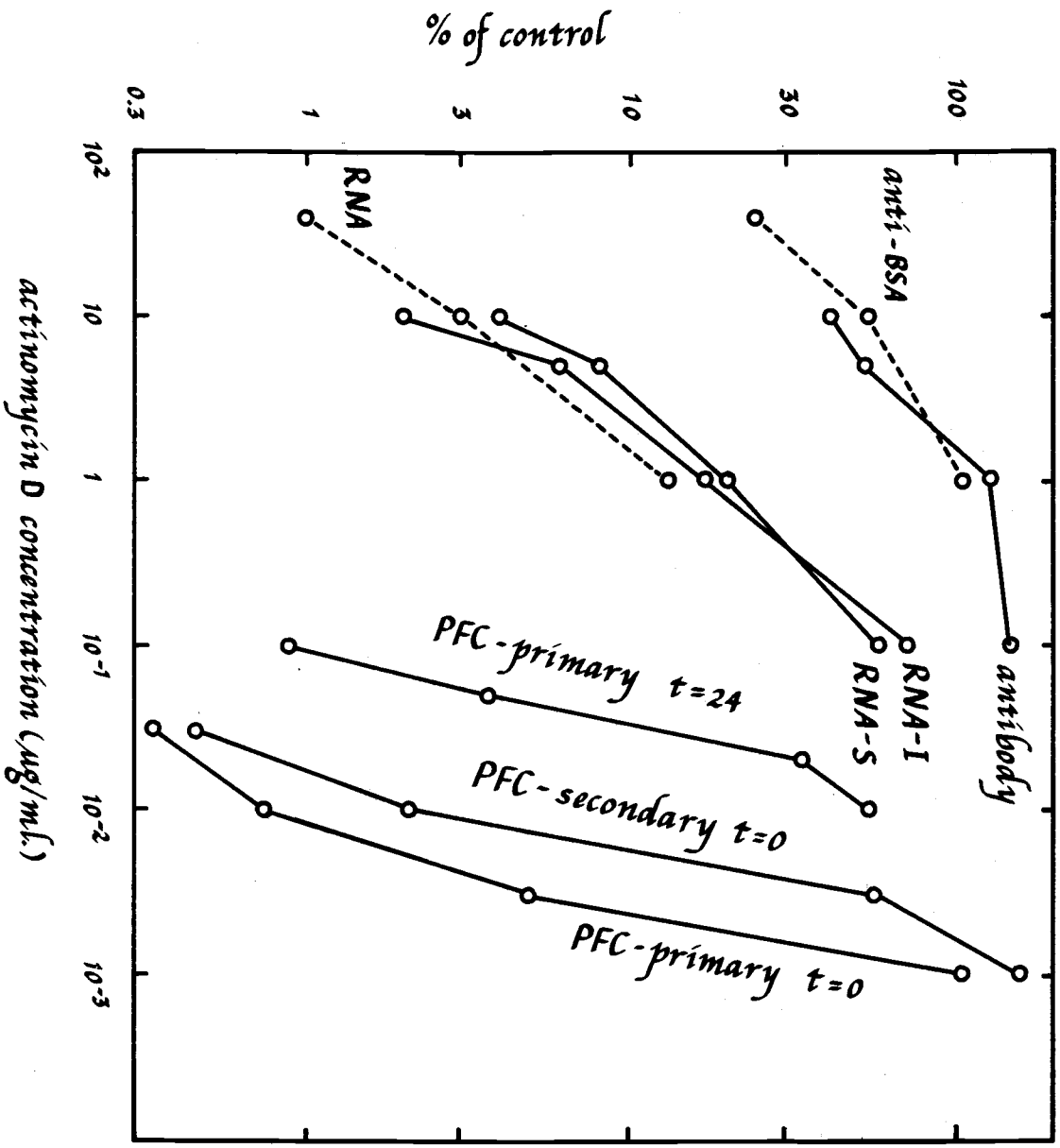


Figure 8. Actinomycin D effects on the production phase of the immune response in short-term culture compared with actinomycin D effects on immune induction.

actinomycin D immediately suppressed RNA synthesis by 80% compared to control cultures, and at the end of four hours of culture the RNA suppression was 95% complete. However the numbers of plaque-forming cells after four hours was still 60% of the control value. This result is similar to that reported by others and implies that, since antibody synthesis does not stop when the RNA synthesis stops, a relatively stable messenger RNA is involved in its synthesis. In the period from the sixth to the twenty-fourth hour of culture the PFC number dropped to less than 5% of the control value. However, Harris found that when this same culture was continued for six or seven days an increase in the number of PFCs occurs, while in the control cultures a decrease in the number of PFCs occurs. Although the total number of plaque-forming cells at the end of seven or eight days of culture are lower than that in the original cell suspension, the PFC value for cultures containing 6.7 μg/ml of actinomycin D may be 10 to 100 or more times as high as the control cultures.

In investigating the nature of this stimulation by actinomycin D (and also for puromycin), Harris found that drug treatments for a period of only 24 hours following two or three days of normal culture in the presence of antigen caused great stimulations in numbers of plaque-forming cells, even up to five times greater than the number of PFCs in the original cell suspension. It was found that all concentrations of actinomycin D from 0.067 μg/ml through 66.7 μg/ml were

effective in bringing about some increases in the number of plaque-forming cells, with the 6.7 μg/ml concentration giving the peak values. Figure 9 shows graphically the course of one of Harris' experiments of this type in which the actinomycin D concentration was 6.7 μg/ml.

The results from a similar experiment from this thesis are plotted in the same manner in Figure 10 for comparison with Harris' data. The data from which this figure is drawn are found in Table 7. In this work the culture system was the same as that used for the induction of the immune responses and the actinomycin D concentration was 1.25 μg/ml. This lower concentration is justified because it falls well within the range of values shown to be active by Harris, and it permits comparison with some of the data from other experiments in this thesis.

Major differences are evident from a comparison of the results shown in these two figures. The culture conditions used in this thesis allowed the number of plaque-forming cells to increase during the culture period, unlike the culture conditions used by Harris. There was no great stimulation of plaque-forming cells in the presence of actinomycin D, but rather a fraction of about 5-10% of the plaque forming cells present at the beginning of the drug treatment period are still detectable as PFCs at the end of the drug treatment. Thus the numbers of PFCs in the actinomycin D treated cultures does

Figure 9. Secondary response initiated in vivo and continued in vitro. Stimulation of PFCs by actinomycin D. (Data of Harris)

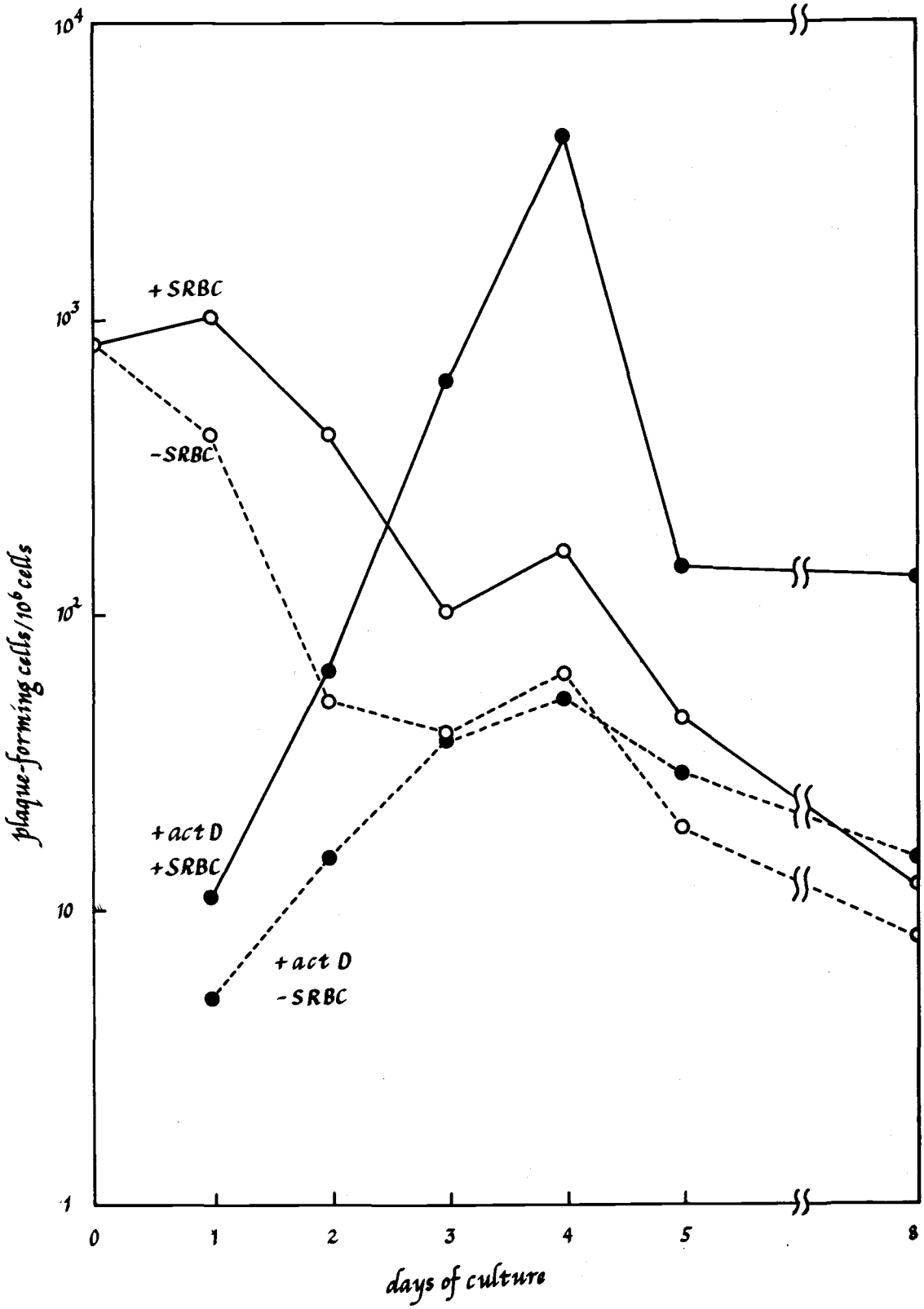
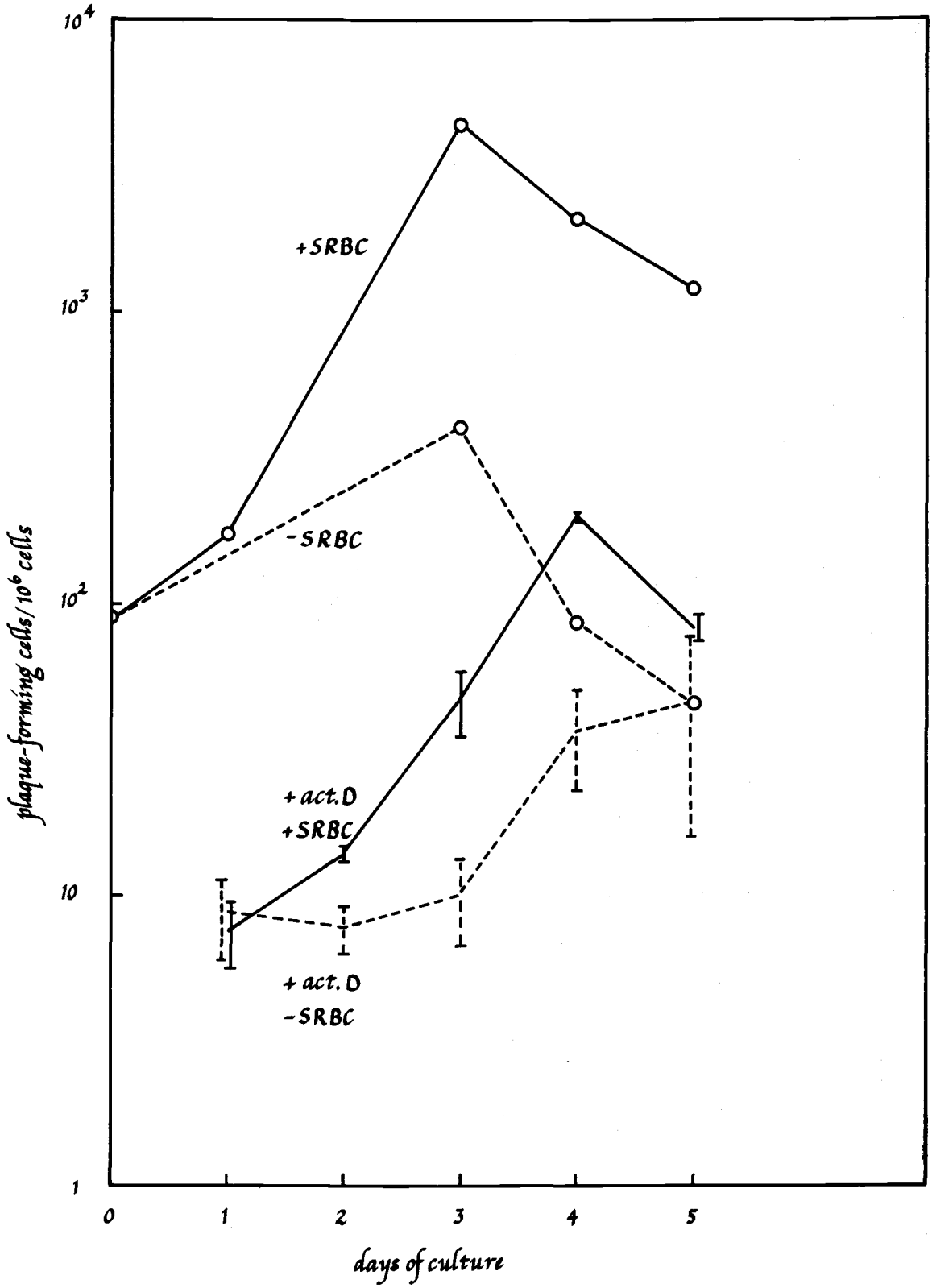

10^4
10^3
10^2
10
1
+SRBC
-SRBC
+act D
+SRBC
+act D
-SRBC
plaque-forming cells/10^6 cells
0
1
2
3
4
5
8
days of culture

Figure 10. Secondary response initiated in vivo and continued in vitro. Absence of PFC stimulation by actinomycin D.


10^4
10^3
10^2
10
1
plaque-forming cells/10^6 cells
+SRBC
-SRBC
+ act. D
+SRBC
+ act. D
-SRBC
0
1
2
3
4
5
days of culture

rise during the course of the experiment, but only because the numbers of PFCs were higher before the drug treatment. Because no stimulatory effects by high concentrations of actinomycin D were seen in this mouse spleen cell culture system, such effects will not be considered further.

Effects of Low Concentrations of Actinomycin D Upon Cellular Functions in Culture

The importance of the synthesis of a specific RNA species in the first hours of the immune induction will be discussed in a later section, but other inhibitory effects of the drug should also be considered as the possible cause of the inhibition of immune induction. Though the synthesis of bulk DNA-like RNA is reportedly not sensitive to very low concentrations of actinomycin D (54, 60), toxic effects, inhibition of cell division, and the inhibition of ribosomal RNA synthesis are manifest in some culture systems at the low concentrations at which the immune induction is inhibited.

In an early assessment of the cytological changes produced in cultures by the action of actinomycin D, Cobb and Walker (15) found that established cell lines such as Hela were much less sensitive to the drug than were primary cultures. Utilizing a primary culture of embryonic beating heart fragments and a range of actinomycin D levels from 10 μg/ml down to 10^{-6} μg/ml the following observations

were made: a) migration and mitosis irreversibly stopped in initial cultures treated for 96 hours with drug concentrations from 10 μg/ml down to 10^{-4} μg/ml, although the cell's beating function was not halted, b) actinomycin D, at 10^{-4} to 10^{-6} μg/ml produced marked cytotoxic changes in five day old cultures after exposure for 48 hours, c) after 48 hours of drug treatment (10^{-2} to 10^{-4} μg/ml) fragments did not grow after removal of the drug. In similar studies with Hela cultures, only slight cytotoxic changes were produced after exposure to actinomycin D concentrations from 10^{-3} to 10^{-5} μg/ml. In general it was found that all cell types in a primary cell population showed some degree of cytotoxic changes and these changes were of a non-specific nature consisting of overall granulation, pyknosis, vaculation and cell death.

In their work on the primary immune response, Bussard and Lurie (12) noted that the viability of drug treated cultures was not significantly different from the control cultures. They proposed that one possible explanation for the inhibition of the immune response by the drug in such systems would be that actinomycin D has a specific toxic effect upon some cells whose presence is mandatory for the immune induction. This type of drug action against specific cell types has been reported in the in vivo immune response by Hanna and Wust (32). In this cytological study of spleen cells from animals undergoing actinomycin D treatment the loss of ability to synthesize

antibodies was correlated to the disappearance of one particular cell type, a large green pyronin-staining cell. This stain is used to preferentially demonstrate the presence of heavy concentrations of RNA in the cytoplasm. This green staining cell probably represents the precursor of the plasma cells, which constitute the bulk of the antibody-producing cells. If indeed the actinomycin D preferentially kills one type of cell which will react to the antigen, the immune response could be effectively halted without the necessity for wholesale killing of the spleen cell suspension. In this regard, however, it should be remembered that the sensitivity of the induction of the secondary response is quite similar to the primary response in spite of the presence of a large number of immunological memory cells present in the cell suspension from immunized mice.

Several investigators have used a system which is quite analogous to some aspects of the immune induction in spleen cells, that of the induction of blast cell transformation in mammalian peripheral leucocyte cultures stimulated by phytohemagglutinin. Jan Steffer (62) showed that doses of actinomycin D as low as 0.0025 μg/ml inhibited this process to the degree that the mitotic index of the drug-treated cultures was less than 1% that of the control cultures. Neu, Leao and Gardner (53) demonstrated in the same type of culture system that actinomycin D (0.02 μg/ml) treatment for 48 hours completely stopped cell division even though the cells were maximally stimulated

to division before the drug treatment. They also reported that this drug treatment produced no alterations of the chromosomes such as breaks or translocation-like cross configurations.

It has been suggested by Arrighi and Hsu (3) that new proteins must be synthesized for the mitotic spindle apparatus at each cell division. The actinomycin D inhibition of mitosis would then be due to the blockage of synthesis of proteins needed for the mitotic spindle. Dutton and Mishell (20) recently reviewed the many experiments showing proliferation in the immune response. The reviewers concluded that proliferation plays a definite role in the development of the antibody-forming cell population. Claflin and Smithies (14) have reported acutal observation of cell division by cells already identified as plaque forming cells. Tannenberg and Malaviya (66), as well as other authors, have demonstrated using autoradiographic techniques that virtually all plaque-forming cells are dividing during the period of logarythmic increase in their numbers. However, using a double label technique in conjunction with the PFC assay, Tannenberg showed that the generation time for the plaque-forming cells was about 13 hours, with the cell cycle divided into about eight hours for the S period, three hours for G1 and two hours for G2 in both the primary and secondary responses. During this period the doubling time for the number of plaque-forming cells is six or seven hours. Thus, he concluded that the PFCs are not all developing from the

division of pre-existing PFCs, and in fact only a small proportion can be accounted for by this process.

In a study with the primary immune response in vitro Dutton and Mishell (19) used a "hot pulse" of tritiated thymidine to selectively irradiate (and thus kill) cells which are in the process of DNA synthesis during the pulse. The authors found that continuous pulsing during the first 24 hours after stimulation by the antigen had little or no adverse effect on the development of PFCs. A pulse of irradiation for a 24 hour period at any time after the first 24 hours, however, caused a severe inhibition of the numbers of PFCs which develop. This indicates that the cells involved in the eventual production of PFCs do not divide at any appreciable extent during the first 24 hour period of culture, but it also indicates that almost all of the precursor cells, as well as the early appearing PFCs are actively dividing at any point in the response after the first 24 hours.

Utilizing a system of in vitro immune induction which was similar to the system of Mishell and Dutton, except for the fact that the antigen was immobilized in agar coating the bottom of the culture dish, Saunders (57) was recently able to enumerate not only the number of plaque-forming cells in the culture, but also the number of clones of cells giving rise to the PFCs. This work demonstrated that all precursor cells which will produce plaque-forming cells are producing cells which do make antibodies by the end of the first 24

hour period. With this system a synchronous coupling of the number of PFCs/precursor unit was evident. The first doubling of PFCs/precursor unit occurred after 17 to 19 hours of culture, and subsequent doubling occurred at seven or eight hour intervals.

Thus several different types of experiments have shown using different in vitro systems that by the end of about 24 hours of culture the initiation steps seem to be complete, and all cells which will produce PFCs are now in a state of rapid proliferation which continues until the population of PFCs has been formed. Since the steps following actinomycin D treatment at 24 hours are not sensitive to 0.01 μg/ml of the drug, it would appear that this concentration does not interfere with the cell's ability to divide in this culture system. Therefore the even greater sensitivity of the induction phase is not due to inhibition of mitosis. The inhibition of the response by higher doses of actinomycin D might well be due in large part to the blockage of cell division, but other inhibitory effects are also active at higher drug concentrations, since great reductions in the number of PFCs occur upon drug treatment even in cultures with high numbers of PFCs before treatment.

Kay and Cooper (39) have studied the effects of actinomycin D on RNA production in the peripheral leucocyte system. It was found that if cultures of lymphocytes previously stimulated by phytohemagglutinin were preincubated with actinomycin D (0.008 μg/ml) for 18

hours and then labeled uridine was added for six hours, incorporation into 28S ribosomal RNA was inhibited by more than 80% while 18S ribosomal RNA continued to incorporate label at more than 50% of the control value. Since current evidence indicates that in mammalian cells ribosomal RNA synthesis occurs as the synthesis of one molecule of 45S RNA which then gives rise to one molecule each of 28S and 18S ribosomal RNA, the differences noted by Kay and Cooper may indicate a preferential decay of one species.

Koch, Trams and Kubinski (41) studied actinomycin D effects in cultures of mammalian amnion cells. They found that the synthesis of ribosomal RNA was inhibited by very low concentrations of the drug (0.005 μg/ml) while the synthesis of messenger RNA continued.

Perry and Kelley (54) have shown in exponentially growing L cells that 18S and 28S ribosomal RNA synthesis is inhibited by much lower concentrations of actinomycin D than is the synthesis of the smaller 4S transfer RNA or the 5S ribosomal RNA. Using a cell concentration of $2\text{-}5\times10^5$ cells/ml they found that synthesis of 18S and 28S ribosomal synthesis continued at 15-30% of the control at an actinomycin D concentration of 0.04 μg/ml, but at a concentration of 0.08 μg/ml the synthesis had fallen below the limits of detection. The synthesis of 4S and 5S RNA species was not decreased by these concentrations of actinomycin D, continued at 80% of the control rate in the presence of 0.4 μg/ml, and was finally inhibited by 90-95% at

a drug concentration of 2.0 μg/ml.

Possible Messenger RNA Formation Important to the Early Induction Stages

Since the inhibition of RNA synthesis is due to the binding of actinomycin D molecules to the deoxyguenosine residues of the DNA (28), the two obvious factors which should influence the actinomycin D sensitivity of a given type of RNA are the length of DNA which codes for its sequence and also the number of G-C base pairs contained in that length of DNA. In the case of limiting amounts of actinomycin D, the longer length of DNA has a much better chance of having at least one bound molecule of actinomycin D than does a short length of DNA with a similar G-C content. The 45S ribosomal precursor RNA is about 200 times the weight of the 4S transfer RNA, and the relative gene sizes are in the same proportion. Although the direct comparison of these RNA species is somewhat clouded by the fact that multiple gene copies for each type exist in each cell, the genes are of such a major difference in length that one could safely expect quite significant differences in the sensitivities to actinomycin D.

Because the antibody messenger RNA molecules must be synthesized during the later stages of the primary immune response, which has a lowered actinomycin D sensitivity, the high sensitivity of the

early stages cannot be attributed to the inhibition of these molecules. The inhibition of other RNA molecules which are needed for the early induction process could account for the observed high actinomycin D sensitivity. If the actinomycin D concentrations in the culture are low enough that one would expect differential sensitivities because of the length of the RNA molecules being produced, it may be possible to speculate as to the size of the needed RNA molecules.

The synthesis of messenger RNA and bulk DNA-like RNA has also been reported to be much less sensitive to actinomycin D than is the ribosomal precursor (54, 60). It is not possible, however, to extrapolate this finding to all messenger RNAs. In very few mammalian protein synthesizing systems have the messenger RNA sizes been well defined. In the case of hemoblobin biosynthesis the probable messenger RNA which was identified is a 10S RNA containing 550 nucleotides (42). In the antibody synthesizing system, the actual antibody messengers are presumed identical with the two rapidly labeled 10S and 13S species found by Kuechler and Rich (40). The light and heavy antibody chain messengers, if monocistronic, can be calculated to require 645 and 1320 nucleotides respectively in order to give the triplet codes for each of the required 215 and 444 amino acids which make up the two chains. These sizes agree well with the species found by Kuechler and Rich in hyperimmune lymph node tissue undergoing a secondary response. The messenger RNA

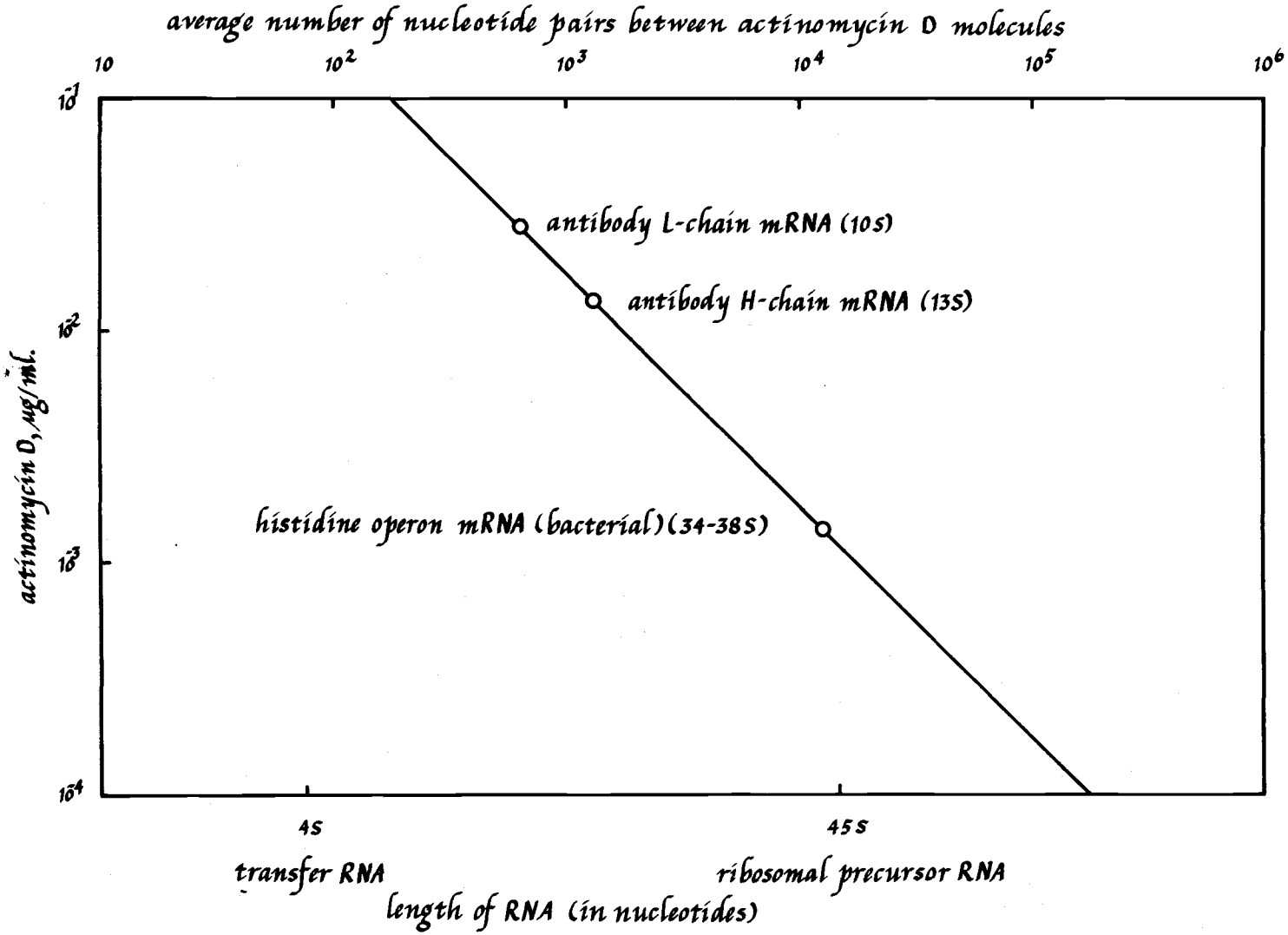
molecules for these "normal sized" proteins are more than ten to 20 times smaller than the ribosomal precursor RNA and would be expected to be less sensitive to inhibition by actinomycin D on the basis of their size alone. It should be remembered, however, that these messengers probably do not have the advantage of multiple gene copies in each cell. As an example of messenger RNA which codes for unusually large proteins, Heywood and Nwagwu (34) have isolated from chick muscle tissue a 25-27S RNA which has been used to direct myosin synthesis in a cell free system. This messenger also appears to be monocistronic, with 6,000 nucleotides needed to code for a single myosin subunit. Some messenger RNA species which are polycistronic, such as the bacterial histidine operon studied by Ames and Hartman (2), are so large as to be placed in the same size category as the ribosomal precursor RNA. The histidine operon was estimated to contain 13,000 nucleotide pairs, and a corresponding 34-38S messenger was detected for this system by Martin (44).

In order to determine whether the concentration of actinomycin D used in the culture system was actually in the correct range where differential effects on the synthesis of these RNA species would be expected, the possible range of numbers of actinomycin D molecules available to each cell was calculated. The actinomycin D concentration of 1 μg/ml is equivalent to 8×10^{-10} moles/culture or approximately 5×10^{14} actinomycin D molecules/culture. At a cell

concentration of 2×10^7 cells/culture this gives a value of 2.5×10^7 molecules available to each cell, which represents the maximum possible number of molecules which can be bound to one cell's DNA. Dutton and Mishell (19) reported that the average nuclear volume for spleen cells was 500 μ^3. This means that in the culture system about 1% of the culture volume is accounted for as nuclear volume. Even if one ignores the drug binding to DNA, 2.5×10^5 actinomycin D molecules would then be present in each cell's nucleus unless it is actively excluded.

The DNA-phosphorus content of each spleen cell has been determined by Menton et al. (45) to be 0.49 picograms, which is equivalent to about five picograms of DNA per spleen cell. From the DNA content of each cell and the maximum number of actinomycin D molecules bound for each cell concentration, the average length of DNA between bound actinomycin D molecules has been calculated. For the 10^{-2} μg/ml drug concentration, the value is 1.8×10^4 nucleotide pairs of DNA between actinomycin D molecules. Figure 11 shows the relationship between actinomycin D concentration and the average length of DNA between drug molecules and also includes some of the points from Table 11 for comparison. Although the actual numbers of bound actinomycin D molecules may be somewhat less than the maximum values used for this figure, the relationship between RNA size and relative actinomycin D concentration should

Figure 11. Relationship between actinomycin D concentration and length of DNA between bound actinomycin D molecules if all actinomycin D is bound. Expected pattern of actinomycin D sensitivity for synthesis of different types of RNA molecules.


average number of nucleotide pairs between actinomycin D molecules
10
10²
10³
10⁴
10⁵
10⁶
10⁻¹
10⁻²
10⁻³
10⁻⁴
actinomycin D, µg/ml.
antibody L-chain mRNA (10S)
antibody H-chain mRNA (13S)
histidine operon mRNA (bacterial) (34-38S)
4S
45S
transfer RNA
ribosomal precursor RNA
length of RNA (in nucleotides)

be useful.

Table 11. Molecular sizes for mammalian RNA types.

RNA type	Protein encoded	Amino acids in protein	S units	Molecular weight	Nucleotides
Ribosomal precursor			45S	$4.1\text{-}4.6\times10^6$	(15,000)
28 S ribosomal			28S	1.6×10^6	
18 S ribosomal			18S	0.6×10^6	
5 S ribosomal			5S		
transfer			4S		77-78
Messenger	Hemoglobin	141 and 146	10S		550
Messenger	Antibody - Light	213-215	10S	2.2×10^5	645
Messenger	Antibody - Heavy	440	13S	3.7×10^5	1320
Messenger	Myosin	2000	25-27S		6000
Messenger	His operon (bacterial)		34-38	4.0×10^6	13000

From this comparison one would expect the RNA species being synthesized in the early induction stages to be several times longer than the antibody messenger molecules. Of course, this may be a precursor-type molecule, which is degraded to smaller RNA molecules before being detected in the cytoplasm.

The role of RNA in the induction of the immune response has not been determined, but the synthesis of antigen specific RNA

molecules and of RNA which complexes with antigen have been detected in the early stages of the induction. Extracts of RNA from immunocompetent cells which have been exposed to antigen have been isolated from non-immunized animals (16, 21, 22). Friedman (24) has shown that a similar increase in the number of plaque-forming cells after treatment with RNA from immunized donor spleens can be completely inhibited if the donor is treated with actinomycin D during immunization. This indicates that a new immunogenic RNA is formed in response to the immunizing antigen. Cohen, Newcomb and Crosby (16) reported that most of the immunogenic RNA was present in an 8-12S fraction. Some reports indicated the presence of an antigen- RNA complex in the RNA preparations used for these induction phenomena (4, 25) and suggested that this complexed form of antigen constitutes a sort of "super antigen." Fishman and Adler (23) later reported that a 7-10S RNA fraction which is free of antigen can induce 19S antibody formation against T_2 phage. These authors also reported a 23-28S RNA fraction which was responsible for inducing 7S antibody formation. Mosier and Cohen (51) recently reported that RNA extracted from spleen or peritoneal cells, following a 30 minute incubation with sheep erythrocytes, could stimulate a rapid increase of about two-fold in the number of background plaque-forming cells in a non-immune suspension of spleen cells. This increase is antigen specific and occurs in less than three hours.

The synthesis of these types of immunogenic RNA in the early stages of induction may account for the extremely low tolerance for actinomycin D during this period. The fact that treatment of the macrophage population alone with actinomycin D was sufficient to inhibit the response is in agreement with this concept, since macrophage preparations were responsible for the synthesis of the immunogenic RNA used in some of the experiments described in the preceding paragraph (4, 21, 22).

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