other types of cells such as macrophages and connective tissue(23), rather than a rise in the level of nucleic acids in the muscle cells themselves.

Corresponding studies with the hybrid strain of the mice gave results similar to those with strain 129.

Summary. Phosphorus in various forms was determined in the subcellular fractions of skeletal muscle from mice with hereditary muscular dystrophy and from their normal littermates as controls. Total phosphorus increased in the nuclear-myofibrillar fraction and decreased in the supernatant, with a net increase for the homogenate. Acid-soluble phosphorus behaved similarly, but did not significantly increase in the homogenate. Lipid phosphorus decreased in mitochondria and increased in supernatant. In the nuclear-myofibrillar fraction, phosphorus of both deoxy- and ribonucleic acids was considerably increased, whereas, in the microsomes, only that of the ribonucleic acid was increased.

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Time Relationship of Injection of Actinomycin D and Antigen to the Immune Response.* (30037)

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We have reported(1) that actinomycin D, given at the same time as antigen (sheep erythrocytes) in the rat, caused a delay in time of appearance of circulating hemagglutinin but had no effect on apparent rate of synthesis or maximal amount of antibody eventually produced at 5 to 6 days. In the mouse, cellular damage, particularly in germinal centers of lymphoid tissue, was most pronounced when the antibiotic was given with antigen(2). Coincident with the cytotoxicity was a delay in appearance of circulating antibody. As restitution and repopulation of lymphoid tissues occurred, hemagglutinin was

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titrated in the serum. In preliminary experiments, the time for injection of the drug to give maximal delay in immune response was found to be 4 to 8 hours before injection of antigen. Brown(3) has reported that actinomycin C given in a single dose within 72 hours before injection of sheep erythrocytes enhanced antibody level in mice bled on day 9 after antigen. It was found also that a suppression of titer occurred when the drug was administered 1 to 4 days after antigen.

The present study was undertaken to investigate further the time relationship of injection of actinomycin D and antigen in both rat and mouse. In the latter, a marked correlation was found between relative delay in appearance of hemagglutinin and degree of cellular damage.

Materials and methods. 1. Animals. Female Sprague-Dawley rats 4 to 5 months of age and male LAF_1 ($C_{57}L$/Cum ♀ × A/He Cum ♂)F_1 mice 15 to 16 weeks of age were used. Mice were kept 10 to a cage and given food and water *ad libitum.*

2. Antigen. Sheep blood was obtained fresh in modified Alsever's solution(4) and washed in Tyrode's solution by alternate suspension and centrifugation at 700 × *g*. Finally, a 10% suspension of erythrocytes (SRBC) was made in Tyrode's solution. Immunization was carried out by intravenous injection of 1 ml of the 10% suspension in the mouse and intraperitoneal injection of 0.5 ml in the rat.

3. Serology. Animals in groups of 4 were selected at appropriate times and killed by decapitation. Blood was allowed to drain for approximately 1 minute, and serum was separated after standing overnight at 2 to 5°C. Serum was stored at −20°C until titrated.

Serial 2-fold dilutions of serum in veronal buffer were made in volumes of 0.5 ml each. To each dilution was added 0.1 ml of a 2% suspension of SRBC, and the tubes were incubated for 1 hour at 37°C and for 16 hours at room temperature. The $\log_2$ titer was determined as the reciprocal of the highest dilution showing hemagglutination.

4. Actinomycin D.† The antibiotic was dissolved in warm 1,2-propanediol (1 mg/ml). Each rat received 50 or 75 μg, while each mouse was given 12 μg. In both groups the route of injection was intraperitoneal.

5. Histologic procedure. Spleen, thymus, and mesenteric lymph node were removed from each mouse and fixed in Bouin's fluid. After sectioning, they were stained in hematoxylin and eosin.

Results. 1. Response in rats. It was of interest to examine the effect of actinomycin when it was given before and after injection of SRBC. Animals were divided into groups of 4, and each animal in a group was injected with 75 μg of antibiotic at the same time as antigen, or 2, 4, 6, 8, 10, 12, 16, 20 and 24 hours after antigen. From previous experience, the difference in titer is most pronounced at 3 or 4 days, and an evaluation of treatment can be made by comparing hemagglutinin titers on these days. Animals were killed, therefore, 4 days after antigen injection, and hemagglutinin measured in the serum. These data are presented in Fig. 1. It can be seen that the treatment produces maximal inhibition with actinomycin when it is given at the same time as antigen. As the time between antigen and drug injections is increased, the effect of the drug is decreased; the drug has little or no effect when the interval between antigen and actinomycin injection is increased beyond 6 hours.

Since 75 μg of the antibiotic given at the same time as antigen produced maximal inhibition, a second set of animals was treated with a lower dose of actinomycin to determine whether the drug would be more effective if it were given before injection of antigen. Fifty μg of antibiotic were given to each animal in groups of 4 at 4, 8, 12, 16, 20, 24, or 48 hours before antigen. Titers found on the fourth day after injection of antigen are presented in Fig. 2. Maximal inhibition was found to be 8 hours before antigen. Relative delay as measured by the difference in slopes of responses extrapolated to the base line was 60 hours. A minimum difference in titer, indicating little, if any, delay in immune response, was noted when the drug was given 20, 24, or 48 hours prior to injection of SRBC.

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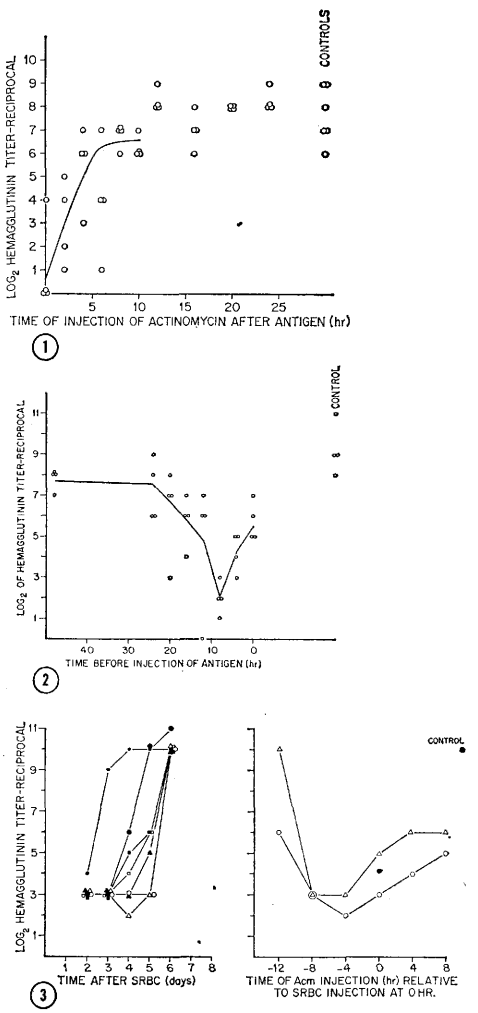

FIG. 1. Effect of actinomycin D on the primary response of female rats to sheep erythrocytes when given after antigen; 75 μg of actinomycin per rat used. All animals were killed on the fourth day after injection of antigen. Each symbol represents one rat.

FIG. 2. Effect of actinomycin on the response of female rats to sheep erythrocytes when given before antigen; 50 μg of actinomycin per rat used. All animals were killed on the fourth day after injection of antigen. Each symbol represents one rat.

FIG. 3. Effect of actinomycin on the primary response of male mice to sheep erythrocytes when given at various time intervals in relation to time of injection of antigen; 12 μg of actinomycin per mouse used. A. Hemagglutinin titers at daily intervals after injection of antigen. Actinomycin given: ● 12 hr before SRBC; △ 8 hr before SRBC; ○ 4 hr before SRBC; ▲ at same time as SRBC; □ 4 hr after SRBC; ■ 8 hr after SRBC. ● SRBC only. B. Titers on the fourth and fifth days after injection of SRBC, plotted as a function of time of injection of actinomycin relative to injection of SRBC given at zero time. ○ titers on 4th day after SRBC; △ titers on 5th day after SRBC; ● SRBC only, titers on fourth and fifth day.

2. Response in mice. The above studies with rats indicated that the time to inject animals with actinomycin for maximal delay in appearance of circulating hemagglutinin was 8 hours before injection of antigen. A similar study was undertaken in mice to establish if the same time relationship existed.

Different groups of mice were injected intraperitoneally with 12 μg of the antibiotic 12, 8 and 4 hours prior to injection of antigen, at the same time as antigen, and 4 and 8 hours after injection of antigen. Four animals from each group were killed at 6 and 12 hours and 1, 1.5, 2, 3, 4, 5 and 6 days after injection of SRBC. Hemagglutinin titers are presented in Fig. 3A. Slopes of the curves, indicative of the rate of synthesis of hemagglutinin(5), are the same when the drug is given before antigen. This slope tends to be decreased when the drug is given simultaneously or 4 or 8 hours after antigen. All groups treated with actinomycin displayed a delayed latent period and the same maximum titers as untreated animals receiving antigen. In Fig. 3B, titers found on the fourth or fifth days of the response are plotted to compare the study in mice with that in rats. It can be seen that the effective time of administration of the antibiotic in relation to time of injection of antigen is approximately the same for both species of animals, that is, 4 to 8 hours before antigen.

Karyorrhexis was a consistent histologic observation in spleen white pulp of antigen-stimulated mice treated with actinomycin. During the early intervals (6, 12 and 24 hours after antigen) in mice receiving actinomycin prior to antigen, large macrophages containing considerable nuclear debris were located primarily in spleen white pulp, germinal centers and scattered throughout the associated lymphocyte mass. By day 2, lymphatic nodules were atrophic and germinal centers were depleted of their characteristic cells.

Mice that received actinomycin simultaneously with or after antigen exhibited the

same pattern of histologic changes; however, the severity of damage and cell loss was less pronounced during the early intervals. In general, degree of damage was directly correlated with relative delay in hemagglutinin titer. This correlation was found during recovery as well. Rate of recovery between days 3 and 6 was greater in animals that received actinomycin 12 hours before, simultaneously with, and 4 or 8 hours after antigen, than in animals that received actinomycin 8 or 4 hours prior to antigen. Repopulation of spleen white pulp germinal centers by the characteristic large basophilic cells was seen in all animals 6 days after antigen. Morphologic counterparts in nonimmunized animals receiving actinomycin only are not severely damaged by the drug (2).

Discussion. Actinomycin is an agent that damages the cell-line during the time that RNA required for differentiation is being synthesized(6-10). Braun *et al*(11) reported that the RNA that controls mitosis is sensitive to actinomycin D only during the first two-thirds of interphase and that the second mitosis does not occur or is delayed by many hours.

The present results indicate that the large basophilic cells implicated in the induction phase of the immune response are damaged when actinomycin is given before, with, or after injection of antigen. The most interesting finding is that the severity of damage is dependent upon the time that the drug is given. When the drug is given 4 to 8 hours before antigen, the second mitotic cycle of many of these cells should be under way at the time of the introduction of antigen. Although mitosis is being inhibited, antigen stimulated the damaged cell. The result is that the cell becomes pycnotic and is phagocytized. When the drug is given 12 hours before antigen, interphase of the second mitosis should be completed at time of injection of antigen. Mitosis is delayed as expected, and presumably antigen cannot stimulate the cell under these conditions. As the cell recovers, it eventually would be stimulated by persisting antigen. We have shown that the relative degree of cytotoxicity and delay in appearance of circulating hemagglutinin is reduced as the time of injection of the antibiotic coincides with or follows time of injection of antigen. It was observed further that while the rate of appearance of such antibody decreased under these conditions, there was no change in maximal amount of hemagglutinin produced.

It is our conclusion that the induction phase of the immune response involves primarily the large basophilic cell of the germinal center. This cell is stimulated to proliferate and differentiate under the influence of antigen. Actinomycin interferes with synthesis of RNA required for such differentiation, and thus metabolism of the cell is altered. A condition of unbalanced synthesis of macromolecular species develops, and the cell is phagocytized.

Effects of actinomycin on the immune response are not limited to the stage of differentiation during the induction phase. It has been reported from studies with actinomycin that messenger RNA is required for production of the immune response(12,13). Cirkovic and Simic(14) found that administration of the antibiotic during the production phase resulted in a slower rate of synthesis, a prolonged latent period, and a lower peak titer. These findings are consistent with those of Brown(3), who appears to have examined the effect of actinomycin on the differentiation or induction phase (antibiotic given in the 72-hour period before antigen) and the effect on the production phase (antibiotic given 1 to 4 days after antigen).

Although actinomycin is known to affect DNA-dependent RNA synthesis primarily, this inhibition and the superimposed stimulation by antigen result in cellular damage and subsequent recovery. The amount of damage is correlated with delay in antibody production and must be considered as a parameter of the response.

Summary. Large basophilic cells in the lymphoid nodule of spleen are damaged when actinomycin D is given before, with, or after injection of sheep erythrocytes. The severity of cytotoxicity is dependent upon the time the drug is given in relation to injection of antigen. Consonant with the relative degree of damage is delay in appearance of circulating hemagglutinin. The time for injection of

animals for maximal delay in appearance of hemagglutinin and coincident maximal cytotoxicity is 4 to 8 hours before injection of antigen.

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Alkaline Phosphatase Activity of L-M Mouse Cells and Variants.* (30038)

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The usefulness of established lines of animal cells for studies in virology, radiation biology, oncology and many other fields is well known. The significance of results obtained with such systems depends, to a large extent, upon the ability to insure the constancy of characteristics of a given line. In a wild population, marked differences may exist among the cells in terms of biochemical characteristics, karyotype, virus sensitivity, etc., as demonstrated by the isolation of clonal strains with widely varying properties. Considerable variability can exist in the "same" strain of cells carried in different laboratories(1) or even as different stocks in the same laboratory(2), depending upon such factors as the choice of medium and conditions of cultivation.

The choice of markers for analysis of population changes depends on a number of factors including stability and ease of determination. Enzymes are particularly suitable for this purpose since they can be studied readily at the cellular level using a variety of histochemical or biochemical methods. One enzyme which appears to be promising is alkaline phosphatase (AP). Nitowsky and Herz(3) in a study of AP in cell cultures of human origin found the enzyme activity in Chang liver cells to be 2-200-fold greater than that in other cell lines studied. After several successive single cell platings of the Chang liver line, clonal strains were derived which showed significant differences in AP activity, and one such strain, morphologically distinguishable by its cell size had an activity only 1/40th that of the parent line.

Fortelius *et al*(4) described a clonal strain of giant HeLa cells which contained little or no AP activity in contrast to the parental line in which the enzyme activity varied from cell to cell but frequently was quite strong.

Studies by Cox and MacLeod(5) indicated that wide variability existed in AP activities among a number of mammalian cell lines and primary cultures. Of 6 uncloned HeLa lines from different laboratories 4 had demonstrable AP activity, and 2 had trace or no activity.

A technique was described by Maio and De Carli(6) in which clonal variants having

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