The Effect of Cytosine Arabinoside upon the Primary Immune Response *in Vitro*[1] (34355)

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The association of DNA synthesis and cell multiplication with the immune response has been the subject of several investigations both *in vivo* and in *in vitro* systems [for a recent review see Ref. (1)]. However, the precise time relationship of DNA synthesis to the progression of the primary response has been a matter of some controversy. The logarithmic rise in hemolytic plaque-forming cells to sheep erythrocytes has been suggested to extrapolate back to time zero with respect to antigenic stimulation (2). To the contrary, evidence from several sources indicate the existence of an initial lag period following antigenic stimulation during which DNA synthesis does not take place (2–5).

Cytosine arabinoside (CA), as an inhibitor of DNA synthesis, has the attractive property of having little or no inhibitory effect upon RNA synthesis, at least in short-term experiments (6, 11). Also, the drug has been reported to enter mammalian cells very fast, insuring rapid attainment of effective intracellular concentrations (7). In the present investigation, cytosine arabinoside was used to determine the time when DNA synthesis becomes essential for the primary induction of hemolytic plaque-forming cells to sheep erythrocytes in mouse spleen organ cultures.

Materials and Methods. Cytosine arabinoside and the various other nucleosides utilized were obtained commercially. The assay for direct plaque-forming cells was that of Jerne and Nordin (8).

The mouse spleen organ culture system used was essentially that designed by Auerbach (9) and described in a previous publication (10). Briefly, mouse spleen fragments in an organ culture system were stimulated with sheep erythrocytes. Fragments showing hemolytic plaque-forming cells in numbers definitely above the background level of non-stimulated fragments were detected on the third day after stimulation. Peak incidence of plaque-forming cells occurred on the fifth day at a mean value of 112 cells/10^6 spleen cells plated.

Experimental Results and Discussion. Determination of dose of CA. In preliminary experiments the concentration of CA sufficient to inhibit the appearance of plaque-forming cells was established. Tissue cultures were set up in media containing graded amounts of the drug. After 30-min exposure of the spleen fragments to the drug, sheep erythrocytes suspended in media containing the respective concentrations of the CA were added. The number of plaque-forming cells were enumerated on the fifth day after stimulation. The results of these experiments are shown in Fig. 1. Each point of the curve represents the mean value of 6 or more cultures. As shown, the presence of CA at the concentration 10^{-5} *M* resulted in 60% reduction in the number of plaque-forming cells. When present at the concentration 10^{-4} *M* or higher, the number of plaque-forming cells were less than 1% of the values of control cultures without the drug. In the subsequent experiments CA was employed in the concentration $10^{-3.5}$ *M* for the purpose of inhibition of a specific immune response. This concentration inhibited more than 90% of DNA synthesis in animal cells *in vitro* (6).

Determination of onset of susceptibility to inhibition by CA. The mode of action of CA (in the form of the triphosphate) is likely to be inhibition of DNA polymerase by competition with cytosine deoxyriboside triphosphate (11). The action of CA can according-

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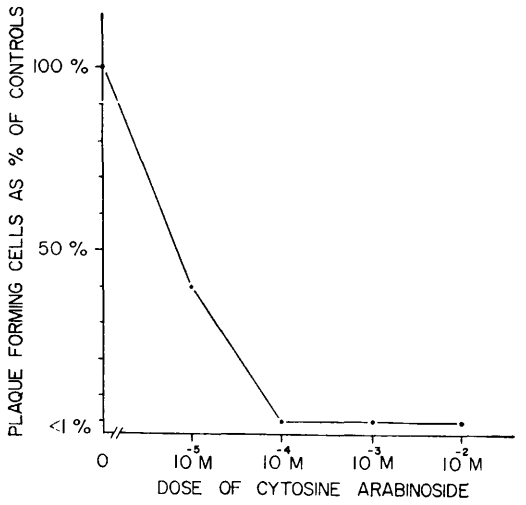

FIG. 1. Dose Response Curve of CA Inhibition of the Primary Immune Response. Mouse spleen fragments were established *in vitro* using media containing graded doses of CA. Control cultures did not receive CA. All cultures were stimulated with sheep erythrocytes and the number of plaque-forming cells was assayed 5 days later.

ly be reversed by addition of excess cytosine deoxyriboside (CdR) (6, 11). In a series of experiments, spleen fragments were established *in vitro* and stimulated with sheep erythrocytes using medium containing $10^{-3.5}$ *M* CA as described above. At various time intervals this medium was removed, the cultures were washed three times with medium without CA, but containing $10^{-2.5}$ *M* CdR and each of the three nucleosides TdR, GdR, and AdR at the concentration $10^{-3.0}$ *M*. Groups of control cultures were included that did not initially receive any CA, but otherwise were treated identically to the experimental cultures. The use of the above nucleoside mixture for reversion of the inhibitory effect of CA was arrived at in a series of preliminary experiments. These experiments showed that incorporation of CdR in the medium at the concentration $10^{-2.5}$ *M* had a slight, but definite depressing effect upon the induction of plaque-forming cells. However, incorporation into the medium of the other three nucleosides abolished this depressing effect. The relative concentrations of nucleosides indicated gave optimal reversion of the inhibitory action of CA.

The results of the main series of experiments concerned with determining the time of onset of susceptibility to inhibition by CA are summarized in Table I. The values in Table I represent the mean values of 6 or more experimental cultures expressed in terms of percentage yield of plaque-forming cells relative to the corresponding set of control cultures. As will be seen, if cultures were given CA at the time of stimulation with sheep erythrocytes, and the action of the drug was reversed 48 hr later as described, there was no (0.03%) recovery of plaque-forming cells when assayed on the fourth day after antigenic stimulation. When the time of reversion was advanced to 24 hr after antigenic stimulation there was a moderate but definite recovery (33%) if the assay was carried out on the third day. However, if the assay was carried out on the fifth day, the experimental cultures yielded plaque-forming cells in numbers comparable to the controls (102%). Because of this improvement of the recovery after an extended period of time, it was not considered to be of interest to carry out assays on reverted cultures later than on the third day. This is the earliest day when a reproducible specific response to sheep erythrocytes is obtained in the system. When reversion from CA inhibition was carried out

TABLE I. Reversion of CA Inhibition of the Primary Immune Response.[a]

Time of reversion of CA inhibition (hr)	Degree of reversion when assayed at the time indicated (hr): 72	96	120
48	—	0.03%	—
24	33%	—	102%
18	63%		
12	96%		

[a] Mouse spleen fragments were established *in vitro* using media containing $10^{-3.5}$ *M* CA and stimulated with sheep erythrocytes. Control cultures did not receive CA. At the time intervals indicated in the left column, the action of the CA was reversed as described in the text. Assays for plaque-forming cells were carried out at the time intervals after antigenic stimulation as indicated and the numbers of such cells expressed in terms of % of the corresponding values of the control cultures.

on the eighteenth or the twelfth hour, the magnitude of the response when assayed on the third day was 63 and 96% of the control values, respectively.

The above experiments indicate that the primary induction of plaque-forming antibody producing cells to sheep erythrocytes is resistant to inhibition by CA and thus independent of DNA synthesis for at least the first 12 hr after antigenic stimulation. By the eighteenth hour, an increasing susceptibility to CA inhibition was evident and if the inhibitor was permitted to act for the first 24 hr, the appearance of plaque-forming cells was reduced by 66%. These data are in general agreement with the view that an initial lag period exists in the induction of the primary immune response during which DNA synthesis is not essential. Similar conclusions have been arrived at by other workers using different experimental systems (3–5). Investigating the effect of thioguanine on the primary immune response in mice, Frisch and Davies (4) noted no interference by the drug when it was administered prior to 18 hr after antigen challenge. Merritt and Johnson (3) found FUdR to be inhibitory to antibody formation to BCG in mice only when given at the eighteenth hour or later after antigen administration. Using death of precursor cells by internal irradiation, due to incorporation of tritiated thymidine in an *in vitro* system, Dutton and Mishell (5) recently concluded that DNA synthesis starts 24–32 hr after antigenic stimulation. This somewhat later onset of DNA synthesis than indicated by our data most likely reflects the fact that a certain amount of radioactive material must be incorporated to kill cells. This sets a certain limit to the sensitivity of this method. Inhibition of DNA synthesis by CA is likely to be more sensitive (7), and thus give more precise information as to the time of onset of DNA synthesis.

Summary. The effect of cytosine arabinoside, an inhibitor of DNA synthesis, upon the primary induction of plaque-forming, antibody-producing cells to sheep erythrocytes was investigated using mouse spleen fragments *in vitro*. It was found: (i) During the first 12 hr, following antigenic stimulation, the immune response proceeded undisturbed by the presence of the drug. (ii) If the inhibitor was permitted to act for the first 18 hr, the number of plaque-forming cells was reduced to 63% of control values. (iii) Extension of the period of action of the drug to the first 24 hr further reduced the number of plaque-forming cells to 33%, and if the inhibitor was allowed to act for the first 48 hr, total suppression of the immune response was observed. These findings indicate that DNA synthesis, essential for the undisturbed progression of the primary immune response as observed in the system employed, is initiated about the eighteenth hour after antigenic stimulation.

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