8. In hamsters implanted with melatonin-filled capsules of various lengths and maintained under an LD 14 : 10 light cycle, exposure to a 50-mm melatonin capsule resulted in the maximum antigonadal effect on the testis. The mean testicular weight of animals implanted with a 50-mm capsule was significantly lower ($P < .001$) than that of animals implanted with a 100-mm capsule. Whether this also represents a dose-dependent differential effect of melatonin on the testis remains to be determined.
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12. This research was supported by PHS research grants HD-03803, HD-00268, HD-07727, and HD-09327 from the National Institute of Child Health and Human Development. We thank Don W. Carroll, Anne Reynolds, and Sam Siddiqui for their assistance.
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Human Interferon Production: Superinduction by 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole

Abstract. *Polyinosinic·polycytidylic acid [poly(I·C)] induced production of interferon by a strain of diploid human fibroblasts (FS-4), measured between 5 and 24 hours from induction, is enhanced up to 128-fold by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a reversible inhibitor of nuclear heterogeneous RNA synthesis. A normalized dose-effect plot shows a close correlation between the superinducing effect of DRB and inhibition of RNA synthesis. Cultures that contained DRB continue to produce interferon for up to 4 days. Removal of the drug at any time during this period leads to a prompt shutoff of interferon production.*

Tamm *et al.* (*1*) reported in 1954 that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was inhibitory for the multiplication of influenza virus. Subsequently mumps virus, vaccinia virus, poliovirus, and adenovirus have also been shown to be inhibited by DRB (*2, 3*). Furthermore, it was observed that DRB inhibits cellular RNA synthesis (*3, 4*). The latter observation has been extended recently by Egyházi *et al.* to the inhibition of RNA synthesis in the salivary gland cells of *Chironomus tentans*, by Tamm *et al.* to L and HeLa cells, and by Granick to chick embryo fibroblasts (*5*). The new evidence indicates that DRB is a selective and reversible inhibitor of nuclear heterogeneous RNA synthesis.

We have used DRB as a probe to study the regulation of interferon production in a strain of diploid human fibroblasts designated FS-4 (*6*). The focus of our studies is the mechanism of the enhancement of interferon production induced by polyinosinic·polycytidylic acid [poly(I·C)] in the presence of inhibitors of RNA or protein synthesis (*7*). Paradoxical enhancement of this kind, usually termed superinduction, has been observed in numerous other systems (*8*). We report that treatment of FS-4 cells with DRB at concentrations that inhibit RNA synthesis leads to an enhanced and prolonged production of poly(I·C)-induced interferon. In contrast to control cultures in which interferon production stops 6 to 8 hours after poly(I·C) induction, cultures treated with 30 μM DRB continue to produce interferon for up to 4 days. Removal of the drug at any time during this period leads to a prompt shutoff of interferon production. The results demonstrate the usefulness of DRB as a tool in the investigation of the regulation of biosynthetic processes and indicate that the interferon superinduction mechanism is closely linked to the inhibition of RNA synthesis.

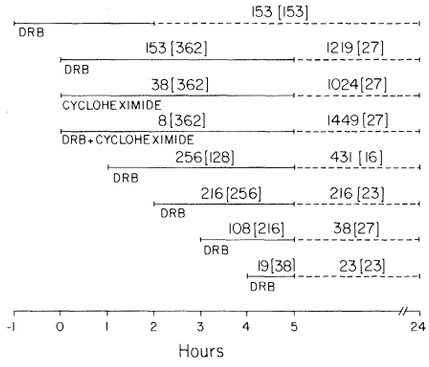
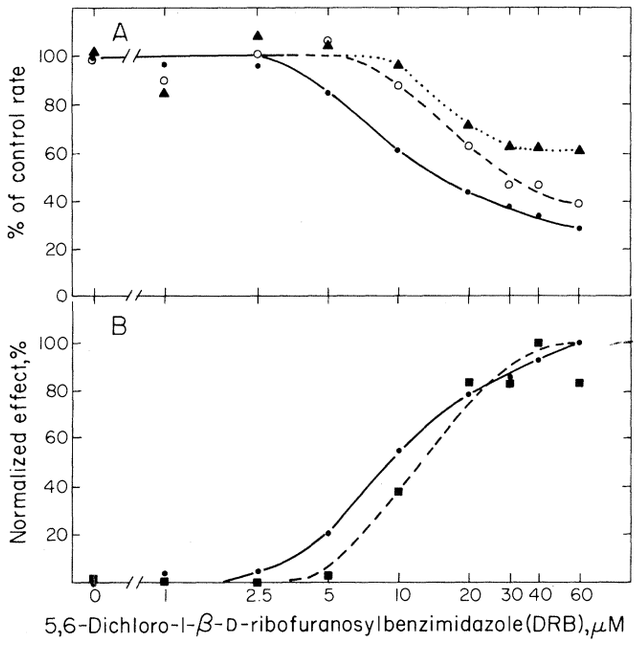


Fig. 1. Temporal characteristics of interferon superinduction by DRB. Cultures (13 days old) in 60-mm dishes were induced with poly(I·C), (100 μg/ml; 2 ml per dish) in Eagle's medium for 1 hour beginning at zero hour; DRB (12.8 μg/ml, 40 μM) or cycloheximide (50 μg/ml, 178 μM) was present during the periods indicated by the solid lines. At the ends of the periods of drug treatment the medium was harvested, and the cultures were washed and replenished with inhibitor-free maintenance medium, which was kept in the cultures during the periods indicated by the interrupted lines. Each number in the figure represents the geometric mean interferon yield in reference units per milliliter from two cultures, where each sample was titrated in duplicate. Interferon yields given outside brackets refer to cultures treated with drugs; those in brackets represent interferon produced in control cultures that were free of inhibitors measured in parallel for the periods indicated. In the groups treated with drugs for 0 to 5 hours, interferon production was measured between 1 and 5 hours.

FS-4 cells were grown at 37°C in Falcon petri dishes (60 mm or 35 mm) in Eagle's medium (*9*) containing heat-inactivated (56°C; 0.5 hour) fetal bovine serum, 10 percent (Grand Island Biological). The cultures were usually used between 9 and 15 days after plating. Poly(I·C) induction was carried out by exposing the cultures to a solution of 100 μg of poly(I·C) per milliliter (obtained from the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) in Eagle's medium for 1 hour, after which the cells were washed four times with warm phosphate-buffered saline (PBS) (*10*). Interferon produced by cells and released into medium was monitored at 37°C in 2 ml (60-mm dish) or 1 ml (35-mm dish) of maintenance medium (Eagle's medium containing 2 percent heat-inactivated fetal bovine serum). Interferon was assayed by a semimicro method (*6*), with FS-4 cells and vesicular stomatitis virus. Interferon titers are expressed in terms of the 69/19 reference standard for human interferon (obtained from the Antiviral Substances Program). Samples that contained inhibitor were dialyzed prior to titration. In all experiments zero time refers to the time when poly(I·C) was added to cultures.

The superinducing effect of DRB on interferon production is illustrated in Fig. 1. In this experiment cultures were treated with 40 μM DRB for varying intervals and at different times with respect to induction with poly(I·C). At 2 or 5 hours, from zero time as defined above, the medium was harvested, the cells were washed four times with PBS, 2 ml of inhibitor-free maintenance medium was added to the cultures, and incubation continued to 24 hours. At this time, the supernatants were again harvested. Control cultures and those containing cycloheximide [50 μg/ml (Polysciences)] were employed in parallel as indicated.

The results show that, whereas control cells produced the bulk of their interferon prior to 5 hours, cells treated with DRB from 0 to 5 hours made a large additional amount of interferon in the 5- to 24-hour period. A reduction in the time of exposure to DRB reduced the amount of interferon made after 5 hours. It is noteworthy that exposure to DRB from 1 to 5 hours was only one-third as effective as exposure from 0 to 5 hours. This suggests that the process, the inhibition of which is responsible for superinduction, may already be occurring during the first hour of poly(I·C) induction. Superinduction by DRB was not additive with that produced



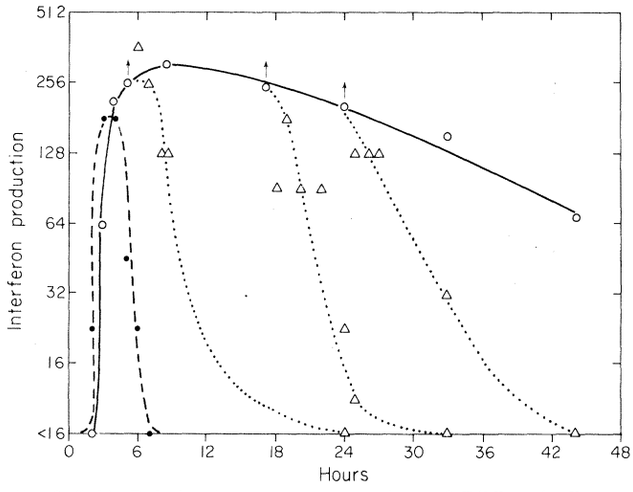


Fig. 2 (left). Relation between concentrations of DRB and effects on RNA and protein synthesis and interferon superinduction. (A) RNA and protein synthesis measured by the incorporation of [^{3}H]uridine and L-[^{14}C]leucine (*11*) 5 hours after the beginning of DRB treatment (*12*). Cellular uptake of [^{3}H]uridine, ○; RNA synthesis, ●; and protein synthesis, ▲. (B) Normalized dose-effect curves of the inhibition of RNA synthesis (●) and of interferon superinduction (■) after treatment with DRB from 0 to 5 hours (*12, 13*). The curve describing inhibition of RNA synthesis is derived from data in (A). Fig. 3 (right). Rate of interferon production (reference units per milliliter per hour) during prolonged treatment with 30 μM DRB and effect of termination of treatment. Four 11-day-old cultures (60-mm dishes) were induced with poly(I·C) in the presence (○), and one in the absence (●), of DRB and the rate of interferon production was followed by repeated medium replacement (2 ml, with or without DRB) and interferon titration. At 5, 17, and 24 hours, one dish, which had contained DRB, was washed four times with warm PBS and the rate of interferon production was from then on monitored in the absence of DRB (△, *14*).

after a 5-hour treatment with cycloheximide. The apparent lack of inhibition of interferon production when the cells were treated with DRB for 3 hours beginning 1 hour prior to poly(I·C) could be the result either of a lack of inhibition of interferon messenger RNA synthesis or masking of a moderate inhibition of interferon messenger RNA synthesis by superimposed enhancement of interferon production. That the latter may be the case is suggested by the greater inhibition of interferon production in the presence of both cycloheximide and DRB as compared to cycloheximide alone.

The effects of DRB at varying concentrations on RNA and protein synthesis (*11, 12*) as measured 5 hours after the beginning of drug treatment are illustrated in Fig. 2A. There was an inhibition of the rate of uptake of [^{3}H]uridine into cells at concentrations of 10 to 20 μM or higher. The rate of RNA synthesis (corrected for the inhibition of [^{3}H]uridine transport) was significantly inhibited at 5.0 μM DRB and was 60 to 70 percent inhibited at a 60 μM concentration of the drug. The moderate inhibition of the rate of protein synthesis at the higher DRB concentrations is probably secondary to prolonged inhibition of RNA synthesis, as it was not observed when protein synthesis was measured 0.5 hour after DRB treatment. The rates of [^{3}H]uridine uptake into cells and RNA synthesis were inhibited to approximately the same extent at 0.5 hour and 5.0 hours after DRB treatment (*12*) (data not shown).

To evaluate the relation between the inhibition of RNA synthesis and interferon superinduction we have constructed normalized dose-effect curves (*13*) of the two phenomena, using data from Fig. 2A. These curves (Fig. 2B) show a quantitative relation between the two processes. The small difference between the two curves at the lower DRB concentrations may reflect a threshold requirement for inhibition of RNA synthesis so as to lead to interferon superinduction.

The reversibility of DRB action and, by inference, the requirement for RNA synthesis for the shutoff of interferon production, is illustrated in Fig. 3. Cells induced in the presence of 30 μM DRB showed a lag in interferon production. This suggests that DRB also has an inhibitory effect on interferon messenger RNA synthesis, but that this effect is less than that on the RNA synthesis, whose inhibition leads to superinduction. Such cells, maintained in DRB, continued to produce interferon for 44 hours. Removal of the drug at any time during this interval led to a prompt shutoff of interferon production. Other experiments have shown that, in the presence of 30 μM DRB, interferon production continues for up to 4 days and decays with an apparent half-life of approximately 12 to 15 hours.

The above observations support the conclusion that the shutoff of interferon production requires RNA synthesis (*7*), presumably nuclear heterogeneous RNA, and also that the mechanism leading to this shutoff may be in operation within the first hour of poly(I·C) treatment. The results also indicate the usefulness of DRB as a probe in studies of regulation of biosynthetic processes.

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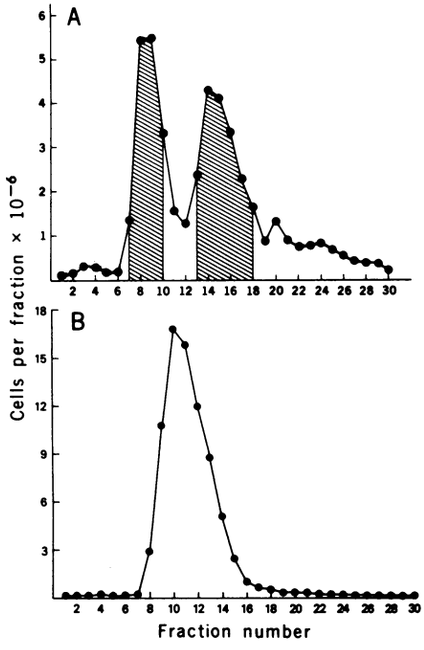
11. RNA and protein syntheses were measured in duplicate cultures (35-mm dishes) at each point by adding 0.25 ml of Eagle's medium containing [5-^{3}H]uridine (250 μc/ml; 21 c/mmole, Schwarz/Mann) and L-[carboxyl-^{14}C]leucine (5 μc/ml; 50 μc/mmole, Schwarz/Mann) to the 1 ml of maintenance medium already present in the dish. After mixing, the cells were incubated at 37°C for 10.25 minutes, rapidly washed ten times with cold (4°C) PBS and solubilized in 1 ml of 1 percent sodium dodecyl sulfate. One milliliter of 20 percent cold trichloroacetic acid (TCA) was then added and the mixture was kept at 4°C for at least 0.5 hour. Samples (10 μl) in duplicate were spotted on Whatman GF/C glass fiber filters and dried, and the radioactivity was counted in a liquid scintillation spectrometer. The remaining sample was filtered through Whatman GF/C filters, and the precipitate was washed thrice with 5 percent cold TCA and twice with ethanol. The radioactivity on the dried filters was measured as above. Radioactivity in the acid-precipitable fraction was corrected for the inhibition of cellular uridine uptake in order to determine the rate of RNA synthesis. It is assumed that there was no alteration in the processing of the precursors after transport into cells.
12. Fifty-four 14-day-old cultures (35-mm dishes) were divided into three groups of 18 each. One group was treated with DRB at varying concentrations in 1 ml of maintenance medium and cellular processes (*11*) assayed after 0.5 hour of the treatment. The other two groups were treated with Eagle's medium containing both poly(I·C) and the appropriate DRB concentration for 1 hour and washed four times with warm PBS; the incubation was continued to 5 hours in maintenance medium that contained DRB. At this time one group was used to assay macromolecular synthesis (*11*) while the other was washed four times with warm PBS and incubated for a further 19 hours. At this time the supernatants were harvested and titrated for interferon.
13. The extent of inhibition of RNA synthesis was normalized with respect to the effect at 60 μM DRB. The increase in interferon titer over the DRB-free control was expressed as a percentage of the maximum increase seen in this experiment at 40 μM DRB.
14. The shutoff curve of interferon production after removal of DRB at 5 hours is based, in part, on data from a second experiment, which are not shown.
15. We thank A. F. Wagner of the Merck Sharp & Dohme Research Laboratories, Rahway, N.J., for a gift of DRB. This investigation was supported in part by research grant AI-03445 and contract NO1-AI-02169 from the U.S. Public Health Service.

24 April 1975; revised 10 June 1975

Hemopoietic Stem Cells in Human Peripheral Blood

Abstract. *A population of lymphocytes, separable from the great majority by virtue of their larger size and their failure to exhibit the rosetting characteristics of thymus-dependent lymphocytes and bursa-equivalent cells, possess true pluripotentiality. On culture in vivo they proliferate and differentiate into erythrocytic, granulocytic, and megakaryocytic progeny. This may be the first clear demonstration of the primitive progenitor blood cell in man.*

An operational definition of the hemopoietic stem cell includes two major criteria: the capacity for potentially unlimited self-renewal and the capability of differentiation into all types of mature blood cells. Experiments on small animals have provided convincing evidence that there is indeed a cell that has these two functions. A mononuclear cell, generally indistinguishable, by light microscopy, from a lymphocyte, has appeared to be the most likely candidate (*1*). Unfortunately, definitive investigative techniques, such as the spleen colony assay (*2*) and total body labeling with tritiated thymidine (*3*), are inapplicable to humans, for technical or ethical reasons. Culture methods in vitro, whether in liquid or semisolid media, preferentially support the growth of progenitor cells committed to differentiation in the granulocyte-macrophage pathway. These cells, which represent some of the early progeny of the true pluripotent stem cell, are demonstrable in normal human peripheral blood (*4*) and have been localized within nonadherent mononuclear cells (*5*). Culture in vivo, in Millipore diffusion chambers implanted intraperitoneally in lethally irradiated mice, supports the growth of unseparated normal human bone marrow and peripheral blood (*6*) and of mononuclear cell concentrates (*7*). By contrast with in vitro methods, this system appears to provide an environment conducive to stem cell proliferation and differentiation (*8*). Our study concerns the growth, in diffusion chambers, of subpopulations of mononuclear cells harvested from the peripheral blood of normal adult volunteers.



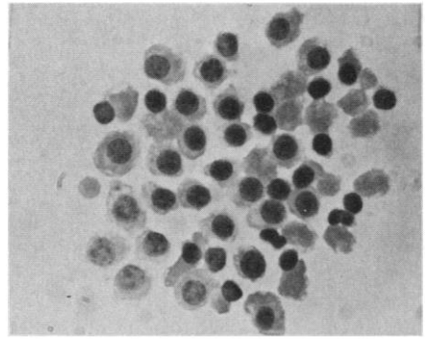


Fig. 1 (left). (A). Separation of non-T mononuclear cells by velocity sedimentation. Increasing fraction number reflects increasing sedimentation velocity and so increasing cell size. The slower peak of smaller cells consists of pure lymphocytes; the second peak is a monocyte concentrate. (B) (lower left). Separation, by velocity sedimentation, of mononuclear cells that fail to form EAC′ rosettes. Fig. 2 (right). Cluster of benzidene-positive late normoblasts harvested at 14 days from Millipore diffusion chamber culture of human hemopoietic stem cell concentrate.

Heparinized whole blood was subjected to isopycnic sedimentation (*9*), and the separated mononuclear cells were depleted of thymus-dependent lymphocytes (T cells) by rosette formation with neuraminidase-treated sheep erythrocytes (N-SRBC) (*10*). When cultured, with or without their accompanying rosettes, the T cells failed to transform. That this was not due simply to inviability was shown by repeating the cultures with immune lymphocytes in the presence of the appropriate antigen (tetanus toxoid or tuberculoprotein). Under these circumstances blastogenesis was marked.

The cells that failed to form these rosettes (non-T cells) were further separated by velocity sedimentation at 1 g in a sucrose gradient (*11*), a procedure that separates cells more on the basis of differences in size than of differences in density (*12*). Two populations of cells were obtained (Fig. 1A): one of pure small lymphocytes (non-T) and the other, a monocyte concentrate of mean composition of 82.7 percent monocytes, 9.9 percent lymphocytes, and 7.4 percent basophils. Culture of the non-T lymphocytes, alone and in combination with cells from the monocyte concentrate irradiated to 2500 roentgens, yielded only lymphocytes, although there was evidence of spontaneous blastogenesis. In contrast, when the monocyte concentrate, containing about 10 percent large lymphocytes, was subjected to culture in vivo, megakaryocytes and all forms of granulocyte precursors were produced on every occasion, and sometimes benzidene-positive normoblasts were obtained (Fig. 2).

In an effort to remove the monocytes, mononuclear cell concentrates were incubated with a complex of SRBC's (E) labeled with rabbit antibody to SRBC's (A) [the 7*S* immunoglobulin G (IgG) frac-