Globin Gene Studies Create a Puzzle

Researchers have three drugs that can turn on fetal globin genes in adults, thus correcting severe anemia, but they no longer think that they know how the drugs work

About a year ago, hematologists thought that they had very cleverly applied basic research in molecular biology to treatment of patients with sickle cell anemia and thalassemia. They treated patients with a drug that, they predicted, would turn on fetal hemoglobin genes that normally are suppressed in adult life. The genes were turned on and the patients severe anemia was partially corrected. The experiment was hailed as a successful effort to bring molecular biology to the bedside (*Science*, 24 December 1982, p. 1295).

But now, the researchers are not quite sure why the genes were turned on. There are theoretical reasons for believing that the drug they used may not be working the way they thought it did and they are finding that two other drugs, which seem to have no ability to unmask suppressed genes, also turn fetal hemoglobin genes on. "I know less now about how fetal hemoglobin is turned on than I thought I knew a year ago," says George Dover, a hematologist at Johns Hopkins University Medical School. "The area is wide open for interpretation." As a consequence, investigators are rethinking not only the mechanisms of the three drugs that seem to turn on fetal genes but also their assumptions about how red blood cells develop and at what time their genetic programs become irreversibly set. They have advanced new hypotheses, supported by data from recent experiments, at scientific meetings.

The idea behind the original experiments was to take advantage of the finding that fetal hemoglobin genes normally are turned off shortly before birth. If these genes can be turned on again in patients with sickle cell anemia or thalassemia, their anemia might be corrected. (This is not necessarily an approach that would work for other genetic diseases. Normally, patients with genetic diseases cannot be helped by turning additional genes on.)

Sickle cell anemia and thalassemia are due to mutations in the β-globin genes, which code for the β-globin subunits of hemoglobin. Adult hemoglobin consists of two β-globin subunits and two α-globin subunits. The hemoglobin made during fetal life consists of two subunits from a gene called γ- and two α-globin subunits. Hematologists knew that if these fetal genes were turned on they would work well in adults because some people with a rare condition called hereditary persistence of fetal hemoglobin make fetal hemoglobin all their lives and have no noticeable clinical effects.

No one knows much about how genes are turned on or off, but molecular biologists did have one clue. Genes that are turned off frequently have methyl groups attached to them whereas genes that are turned on do not have methyl groups on them. The drug 5-azacytidine, which is used to treat leukemia, prevents the incorporation of methyl groups into newly synthesized DNA. Joseph DeSimone, Paul Heller, and their colleagues at the University of Illinois College of Medicine reasoned that 5-azacytidine might conceivably hypomethylate fetal globin genes and thereby turn them on.

Investigators are rethinking the mechanisms of the three drugs that seem to turn on fetal genes and their assumptions about how red blood cells develop.

They tried the experiment in baboons and it worked. Fetal hemoglobin was produced. Then they collaborated with Timothy Ley, Arthur Nienhuis, and their associates at the National Heart, Lung, and Blood Institute (NHLBI) in experiments with patients. Independently, Dover and his colleagues tried the same thing. It worked again. The patients' fetal hemoglobin genes were turned on and, surprisingly, there was no evidence that other genes were also turned on. No one knew why the drug should be so specific, but they speculated that perhaps they were missing the drug's effects on other genes. Their assays, they suggested, were too insensitive. But the drug's apparent specificity was the least of their problems. The more the researchers thought about this experiment, the more the data troubled them.

The problem was that the new expression of fetal genes occurred too rapidly. "The effect is *extremely* rapid," says Dover. "You get a response within 24 to 48 hours after a single dose of the drug." But for 5-azacytidine to remove methyl groups from fetal hemoglobin, it has to work in dividing cells. "There is very little time for cells to divide after the first dose of the drug," Dover says. David Nathan, a hematologist at Harvard Medical School, agrees. "I was deeply skeptical of that [hypomethylation] role," he says.

One hypothesis that occurred to Nathan and to George Stamatoyannopoulos and Thalia Papayannopoulou of the University of Washington School of Medicine was that 5-azacytidine acts not by hypomethylating but by stopping the growth of dividing cells. It is one of a large group of drugs known as S phase specific cytotoxic agents, which means that they arrest cells in the so-called S phase of the cell cycle when they divide. If it is this particular toxicity of the drug that somehow results in an increased synthesis of fetal hemoglobin, then other S phase specific cytotoxic agents should have the same effect, whether or not they also hypomethylate. If so, Nathan points out, then researchers will have a whole new class of drugs to treat sickle cell and thalassemia patients.

Hydroxyurea and cytosine arabinoside, two other drugs used to treat cancer, are among those that stop the growth of dividing cells. Nathan and Norman Letvin of the New England Regional Primate Research Center in Southborough, Massachusetts, decided to try hydroxyurea in monkeys to see if it causes an increase in fetal hemoglobin synthesis. It does. Stamatoyannopoulos and Papayannopoulou tried cytosine arabinoside in baboons and found that this drug, too, causes an increase in fetal hemoglobin synthesis.

Moreover, when Nathan and his associates compared the amount of fetal hemoglobin made and the timing of the fetal hemoglobin synthesis in monkeys given either of the two drugs, they found that the results were nearly identical. Stamatoyannopoulos and Papayannopoulou found that the response in ba-

boons given cytosine arabinoside is exactly the same as baboons given 5-azacytidine. Nathan concludes that the three drugs "probably act in the same way. Methylation has nothing to do with it."

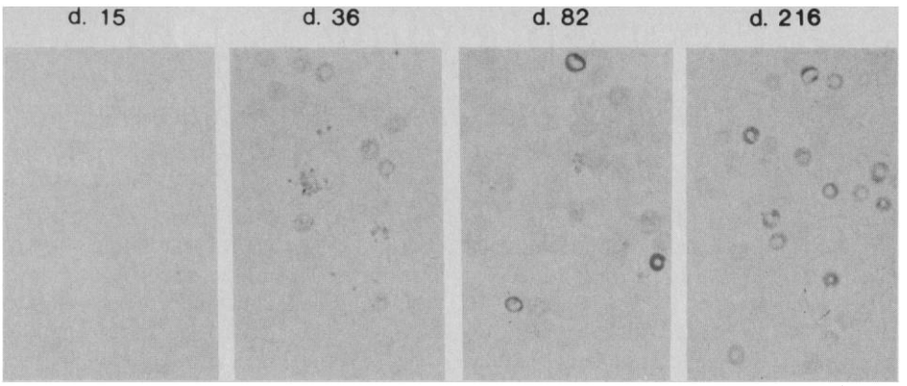
But then what *does* account for the drugs' effect? Nathan proposes two hypotheses, Stamatoyannopoulos suggests another, Dover disagrees with Nathan and Stamatoyannopoulos about one of them, and molecular biologist W. French Anderson of the NHLBI argues that methylation still might be playing some role.

One of Nathan's hypotheses is based on information from in vitro cell culture systems. In these systems, very primitive red blood cell progenitors can be made to differentiate quickly, skipping normal stages of development. When this happens, they make more fetal hemoglobin. When a patient gets hydroxyurea or 5-azacytidine, Nathan reasons, the drug should profoundly affect mature cells in the bone marrow which are rapidly dividing prior to becoming reticulocytes—the fully differentiated cells that circulate through the body and do nothing but make hemoglobin. These mature cells would stop dividing and, Nathan says, "more cells will arise from progenitors." But with this rapid demand for new mature cells, the early progenitors will be forced to differentiate rapidly, and thus will end up making a great deal of fetal hemoglobin.

In support of this hypothesis, Nathan finds that 5-azacytidine and hydroxyurea kill or arrest the development of mature progenitors in the monkey. Stamatoyannopoulos and Papayannopoulou, who agree with this hypothesis, report that 5-azacytidine and cytosine arabinoside kill mature progenitor cells in baboons.

Dover, however, does not go along with this proposal. "This is where David Nathan and I part company," he says. If the drugs were acting the way Nathan proposes, Dover remarks, then you would expect to see evidence that mature bone marrow cells are suppressed in patients who are given lower doses of the drugs than the animals are given. "We tested that theory. We looked at bone marrow cells from patients taking 5-azacytidine and we did not see any evidence that erythroid cells were being killed," Dover says. "The lore about these drugs killing cells is misinterpreted."

Nathan, however, says he is not wed to the hypothesis about progenitor cells. The problem with the idea, to his mind, is that fetal hemoglobin appears so quickly that "it is hard to imagine that it is entirely due to effects on progenitor cells." Another—and even more speculative possibility—is that hydroxyurea and 5-azacytidine delay or set back a normal switching mechanism that cells use to turn off fetal globin genes and turn on β-globin genes. It was always believed that late erythroid cell precursors already have their genetic programs set—they are programmed by that time to make only α-globin and not γ-globin. Now, says Dover, "We have to reexamine that whole concept."



Fetal globin genes turned on

Monkey red blood cells normally contain virtually no fetal hemoglobin, which stains them dark gray in these photographs. On day 15, before the experiment began, the monkey blood cells are very pale gray. On day 36, after the monkeys are made anemic by bleeding, they make a small amount of fetal hemoglobin. The fetal hemoglobin increases on day 82, which is 6 days after a course of treatment with hydroxyurea. By day 216, 6 days after a sixth course of hydroxyurea treatment, there is substantial fetal hemoglobin in the cells. [Source: Norman Letvin and David Nathan]

Still another possibility, Stamatoyannopoulos suggests, is that the drugs act directly on the early progenitor cells, stimulating them to differentiate rapidly. When he treats animals with cytosine arabinoside or 5-azacytosine for only a short time, he says he does not see a killing of late progenitors, but he does see what he describes as "a *tremendous* recruitment of early progenitors." How the drugs might act on the early progenitors is a complete mystery.

In the meantime, Anderson and his associates have looked in a cell culture system to see whether 5-azacytidine does in fact hypomethylate fetal globin genes. They find that it does and that it hypomethylates these genes selectively. Other genes, such as insulin genes and an oncogene are remethylated shortly after they lose their methyl groups. The fetal globin genes are not. And when the fetal genes lose their methyl groups, they are expressed. Anderson is not yet sure why the globin genes are permanently hypomethylated nor does he know how to reconcile the hydroxyurea and cytosine arabinoside work with the methylation hypothesis. No one has ever looked to see if hydroxyurea or cytosine arabinoside can hypomethylate, but molecular biologists have always assumed they cannot. Anderson, for one, is now going to test hydroxyurea to see if it, too, affects methylation.

It may be, of course, that more than one thing is going on and that hypomethylation, for example, causes the persistent effects of the drugs—the fetal hemoglobin is produced for a few weeks after a single dose of the drugs—whereas other mechanisms account for the drugs' immediate effects. But whatever the mechanisms turn out to be, hematologists have, for now, a promising way to treat patients with sickle cell anemia and, possibly, thalassemia.

Dover cautions, however, that just because the drugs cause the production of fetal hemoglobin does not necessarily mean that they are useful therapeutically. The drug 5-azacytidine is a carcinogen, at least in the high doses used to treat leukemia. No one knows whether it will cause cancer in the much lower doses used to treat persons with sickle cell anemia. Clinical studies of hydroxyurea are just starting, and no one yet knows how effective a treatment it will be for these inherited anemias. And, Dover points out, the only way to really learn whether the drugs relieve the symptoms of the diseases is with a controlled clinical trial.

Nathan, Nienhuis, and Dover are now planning to collaborate in a clinical trial of hydroxyurea in sickle cell anemia patients. However the trial turns out, these investigators think they are bound to learn something about how blood cells normally develop and about how genes are turned on and off. If so, they will be more than compensated for their initial disappointment that the original explanation of 5-azacytidine's actions was at least too simple and possibly incorrect.—**GINA KOLATA**