STADLER SYMP. Vol. 8 (1976) University of Missouri, Columbia
pp. 171-180

THE DEVELOPMENT OF NEW DRUGS FOR GENETIC DISEASES

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SUMMARY

The genetic diseases of man constitute a unique challenge to the medical scientist. For the past several years our laboratory has been developing new drugs for two of the more common genetic diseases—sickle cell anemia and thalassemia. The status of the development of sodium cyanate as the first chemometallactic agent for the treatment of sickle cell anemia and the development of new iron chelating agents for the treatment of iron overload in patients with thalassemia are discussed.

INTRODUCTION

Genetic diseases of man present an important challenge to the medical scientists of the future, not only for developing the means of dealing with them, but most importantly because of the recent realization of the magnitude of the numbers of people afflicted. The once thought of rare genetic diseases are not that rare, and, in total, constitute a major health problem in our society. For example, the aggregate of all genetic diseases composes the majority of pediatric hospital admissions in North America (1,2). The burden of these diseases to the patient, the family and the society is considerable, both economically and emotionally. It is apparent that something must be done, and the flurry of congressional laws earmarking federal monies for specific genetic diseases attests to the concern of the society. The need for specific legislation is worthy of comment.

At the present time the development of new drugs in the United States is carried out nearly exclusively by the drug industry. These companies must, by their nature, consider the costs of developing new drugs with the possibility of recouping these expenses plus profit sometime in the future by the sale of the drug. The relatively small numbers (less than one million in the industrial world) for any individual genetic disease thus restrict the amount of money invested by the pharmaceutical

industry. By default, this leaves the responsibility for developing new drugs in the domain of the National Institute of Health. The NIH has not accepted this responsibility readily for several reasons. Firstly, the organization has not been involved in new drug development and lacks expertise. Secondly, the bureaucratic response is usually stated that no one knows what to do. Third, and most importantly, the NIH is extremely sensitive and vulnerable to publicity attendant upon a potential side effect from a new agent. Congress, under pressure from lay groups, by earmarking money is, in effect, forcing the NIH to assume the responsibility that it has neglected.

For the last several years our laboratory has been involved in developing stratagems for ways to deal with genetic diseases. Realizing that genetic engineering was some distant time in the future, we have attempted to take advantage of the advances in chemistry and biochemistry that have occurred in the past 25 years. The first disease that we investigated was Sickle Cell Anemia (3,4). Our approach was to determine the feasibility of modifying the abnormal gene product hemoglobin S so that it would function more normally by reacting it with a small molecule, cyanate. Although the approach of modifying gene products had been considered many times, cyanate represents the first practical example of a chemometallactic agent.

SICKLE CELL ANEMIA

Patients with sickle cell anemia are homozygous for the gene hemoglobin S and number about two million worldwide, with most living in Africa (5). The carriers or sickle cell trait individuals have a mixture of hemoglobin A and S in their red cells, are not symptomatic, and are believed to be more resistant to death during childhood from malaria than children having only hemoglobin A. This latter advantage has led to the fixation of this gene in large populations living in regions of Africa and the Mediterranean where malaria is endemic. The proportion of the population with sickle cell trait in malaria regions can be very high and has been recorded at 40% in Central Africa. In the United States 10% of Blacks are carriers for hemoglobin S. The principal points of the clinical picture of patients with sickle cell anemia can briefly be described as moderate to severe anemia, progressive organ damage throughout the body, and episodes of severe pain, termed sickle cell crisis. In the United States many patients die in the first few decades of life, although a few survive beyond the age of fifty. Patients in Africa die much younger and are especially susceptible to death from malaria. The crisis accounts for much of the morbidity associated with the disease process; it is not uncommon for a patient to have 5 to 20 hospital admissions during a year. At the present time, the treatment of the crisis consists of hydration and relief of the pain until the crisis subsides.

Insight into the pathophysiology of sickle cell anemia came in 1927 when Hahn and Gillespie (6) observed that the red cells from a patient with sickle cell anemia assumed a variety of bizarre shapes following oxygen removal. This sickling of the

cells reversed upon the reintroduction of oxygen. Thus the pathology associated with sickle cell anemia is believed to be due to the sickling of the cells that occurs in the capillaries when the red cells give up oxygen to the surrounding tissues. In addition to their bizarre shape, the sickled cells develop a rigidity that further interferes with their passage through the capillaries. This rigidity results from the aggregation of hemoglobin S molecules which form a liquid crystal in the structure and push the cell into abnormal shapes. If the sickled cell is trapped in a capillary, it blocks the passage of other red cells which, in turn, sickle when they give up their oxygen. If the sickled cell is dislodged, it returns to a normal shape in the lung when oxygen is again reintroduced. However, if the sickled cell does not move, the red cell is destroyed and the surrounding tissue is impaired and possibly dies from the micro-infarction. The sickling-desickling scheme is constantly occurring and accounts for the anemia and progressive organ damage that occurs in the patient. Although it is assumed that the clogging of capillaries is involved in the pain of the crisis, it is not understood what precipitates these episodes.

The next important advance in the understanding of sickle cell anemia came in 1949 when Pauling et al. (7) were able to show that the underlying cause of the sickle cell anemia was the presence of an abnormal hemoglobin in the erythrocytes of the patient. This hemoglobin has the unusual property *in vitro* of forming reversible gel-like aggregates on deoxygenation. Several years after these observations, Ingram (8) was able to demonstrate that the only difference between hemoglobin A, the normal adult hemoglobin, and hemoglobin S is a single amino acid substitution at the sixth position of the β-chain. Hemoglobin A has a glutamic residue, while hemoglobin S has a valine. All of the other amino acids in the β-chain and the amino acids of the α-chain are identical.

The discovery (3) of the antisickling properties of cyanate was based on the hypothesis that cyanate was the active agent responsible for the improvement of patients treated experimentally with massive doses of urea. It now appears that this hypothesis was erroneous since massive urea therapy has not been shown to ameliorate the outcome of crisis, and the amount of cyanate present in urea infusions is not enough to significantly carbamylate the hemoglobin. On the other hand, the hypothesis did lead to the identification of cyanate as an agent that inhibited red cell sickling *in vitro* and lengthened the survival *in vivo* of sickle cell erythrocytes. The antisickling action of cyanate is presumably due to the specific irreversible carbamylation *in vitro* and *in vivo* of the amino-terminal valines of both the α and β-chains of hemoglobin (4). The specificity for these functional groups is believed to lie in their similarity in structure and reactivity to carbon dioxide. Both of these small molecules react with the terminal valine residues of hemoglobin. The important difference is that the CO_2 reaction is reversible, while the cyanate reaction is irreversible. The specific and irreversible nature of the carbamylation reaction with hemoglobin permits the achievement *in vivo* of approximately 0.5 carbamyl groups per mole of hemoglobin tetramer without

serious side effects.

After the initial finding of the antisickling properties of cyanate, an evaluation of the compound was undertaken to determine its potential usefulness in the treatment of patients with sickle cell anemia. First, it was necessary to show that the cyanate had no adverse effect on the red blood cell (9). Then a lack of toxicity in several animal species had to be documented (10). The incubation *in vitro* of red cells with cyanate was not found to be deleterious to the metabolism of the cell, although the affinity of the hemoglobin for oxygen increased in proportion to the amount of cyanate that had reacted. It is believed that the increase in oxygen affinity is in large part responsible for the antisickling property of the drug. Although it was first feared that the increased affinity of hemoglobin would create a "functional anemia" in these patients, experimentation in animals has revealed that the high oxygen affinity blood can deliver oxygen without impairing the function of the tissues (11). Over the last few years cyanate has been evaluated extensively in a number of experimental animals. At moderate doses of the drug, the most striking observation is the increased oxygen affinity of the blood of all the species that have been studied.

In order to assess the ability of cyanate to inhibit red cell sickling *in vivo*, the following experiment was performed (12). An aliquot of blood was taken from the patient and incubated *in vitro* with sodium cyanate. The red cells were then labelled with ^{51}Cr and the blood sample reinfused into the patient. At regular intervals an aliquot of blood was removed and the amount of remaining radioactivity in the blood determined. This procedure gives an assessment of red cell survival *in vitro*. It was found that this survival was significantly increased in all the patients that were studied compared to the value obtained if the cells were not treated with cyanate. This increase in the red cell survival has since been shown by a number of investigators (13,14).

The next step in the development of cyanate was to determine how to take advantage of the carbamylation reaction. There are two major possibilities. The first is to withdraw a quantity of blood from the patient, react the red cells with cyanate, wash the cells free of unreacted cyanate and return the blood to the patient. The second method is to administer the drug orally. Each method has obvious advantages and disadvantages. The extracorporeal treatment is the safest because very small amounts of unreacted cyanate would enter the body and cause undesirable side effects. However, this procedure is time-consuming and expensive and can benefit only a relatively small number of patients. Of the estimated two million people in the world with sickle cell disease, only a small minority would have access to the necessary facilities. The oral administration of the drug does offer a way of attacking the problem. Initial clinical studies (15) revealed that it was possible to achieve carbamylation by both routes without significant side effects. We decided to further evaluate the oral administration of the drug, and Dr. Diederich of Kansas City decided to further

evaluate the extracorporeal method.

The introduction of the carbamyl group onto the hemoglobin S molecule has been observed to dramatically alter the hematological picture of the patient (16). Following carbamylation by either method, the red cells in the circulatory system live longer, and as a result, the total number of red cells in the circulation is increased. The amount of increase varied from patient to patient but was significant in most patients. In addition, the number of new cells in circulation decreased, indicating that the production of these cells by the bone marrow had been reduced to a more normal level.

Fig. 1 records the hematological response of a patient receiving oral cyanate therapy. It can be seen that as the amount of carbamylation increases, the amount of hemoglobin increases to near normal levels, while the number of reticulocytes and the bilirubin decrease.

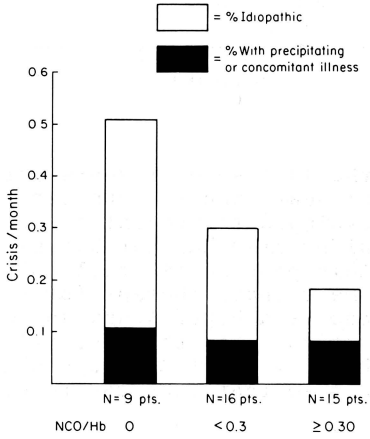

Fig. 1. The effect of cyanate on the frequency of crisis. The light area represents crises of unknown origin and the shaded area represents crises associated with an identifiable precipitating factor or concomitant illness.

In addition to the hematological response, both studies have revealed that carbamylation of the hemoglobin results in a significant decrease in the frequency of painful episodes that occur. It is of interest that the most significant group of crises that are prevented by carbamylation are the crises of unknown origin. The cause for this is not understood, although it points to a different pathophysiology for these two types of crises. Further investigation is needed to delineate the exact effect of cyanate on the frequency of crises.

Unfortunately, the oral administration of cyanate had to be stopped when two side effects appeared in the patients receiving the drug. The first was a peripheral neuropathy which was clinically evident in two of the patients (17); and, second, cataracts, which also occurred in two patients (18). Both of these deleterious side effects reversed when the administration

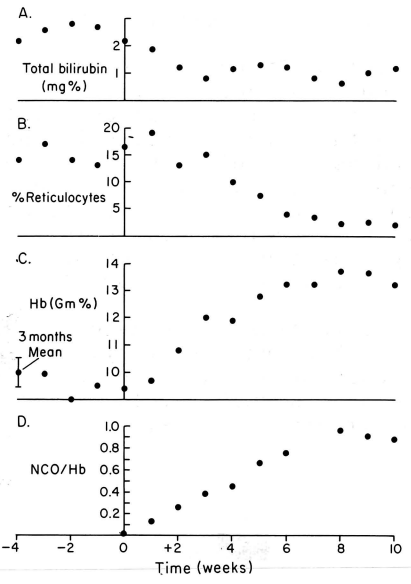

Fig. 2. Response of a patient on 43 mg/kg/day oral sodium cyanate. Panels ABC and D record the changes in bilirubin, reticulocytes, hemoglobin, and carbamylation, respectively.

of the drug was stopped. For the last several years animal studies have been undertaken to delineate the reasons for the toxicity and, hopefully, methods developed to prevent these forms of toxicity. The extracorporeal program has not had significant problems in toxicity, and this program is still being continued (19). At the present time cyanate therapy for sickle cell anemia can only be described as a hopeful new approach awaiting further clinical evaluation. Nonetheless, it has opened the door for at least thinking of other drugs which would react with abnormal gene products so that they can function more normally.

THALASSEMIA

The second genetic disease that we have been actively trying to develop new therapeutic regimens for is thalassemia. The thalassemias are, in fact, a class of genetic diseases in which there is an imbalance in globin chain synthesis (20). Thus in β-thalassemia the blood cells cannot produce enough β-chain of the hemoglobin molecule, whereas in α-thalassemia there is an inability to synthesize the appropriate amount of α-chain of the globin molecule. It is estimated that 3 million in the world are afflicted with a form of thalassemia that has clinical manifestations. The geographical distribution of these diseases is roughly from the Mediterranean basin where β-thalassemia occurs through the Middle East and to the Far East where α-thalassemia is extremely prevalent. The thalassemias, like hemoglobin S, are believed to have conferred to the hetereozygotes a genetic advantage for living in malaria regions.

The gene frequency can run as high as 2-30% in these regions.

Because of the problem of producing hemoglobin, the patients can have anemias that run from very severe (hemoglobin < 3g/100ml) to moderate (hemoglobin ∿ 6-7g/100ml). The severe anemia of patients with homozygous β-thalassemia is not compatible with life, and the children who are born normal with fetal hemoglobin usually die in the first two years of life after the switch to the adult hemoglobin is made. The homozygotes of α-thalassemia cannot produce hemoglobin F and die in utero. The heterozygotes of α-thalassemia have mild to moderately severe anemia. In order to maintain life in severely anemic children, blood transfusion therapy is carried out at regular intervals. It is now possible with this regimen to maintain these patients until the age of approximately 20 when the patients succumb to the pathological changes induced by iron overload. Since the body lacks an effective means of excreting iron, virtually all of the iron administered as transfused red cells is retained in the body and stored in various tissues as ferritin or hemosiderin. This accumulation of iron, particularly in the heart, liver and pancreas, leads to progressive fibrotic changes in these tissues, resulting in organ failure and early death. The accumulation of iron and resulting pathological changes also occur in the anemic thalassemia patient who is not transfused; this is presumably due to increased iron absorption from the diet.

One of the obvious ways to deal with the problem of sickle cell anemia and β-thalassemia is to get the patient's blood production system to switch back to the synthesis of fetal hemoglobin. Unfortunately, at present there is no clue as to how to induce this switch. While other workers search for clues, we decided to embark on a program to design new iron chelating agents to prevent the accumulation of iron and, hopefully, the early demise of these patients. Previous to our work, an iron chelator, desferrioxamine, had been proposed as a therapeutic agent which has been evaluated in England where it was found to retard the accumulation of iron in these patients. One of the major drawbacks for its use has been that it is not orally absorbed and has to be injected intramuscularly once per day. This is not only an inconvenience but is not practical in poorer regions of the world. Accordingly, our program has had particular emphasis on those agents which would be orally effective.

Initially our investigation focused on compounds derived from specific iron chelators produced by microbes (21,22). Because the iron in soil exists almost exclusively in relatively insoluble oxides ($FeOH_3$, $K_{sp} = 10^{-38}$), microbes have developed iron transport systems which utilize chelating agents in order to obtain iron from their environment. These chelators are either hydroxamic acids (23), of which desferrioxamine is an example, or conjugates of 2,3-dihydroxybenzoic acid (2,3-DHB) (24). 2,3-DHB is found conjugated with such compounds as glycine, serine, lysine, and spermadine. Over the past few years we have evaluated a number of natural products and synthetic compounds for tneir ability to induce iron excretion in rats that

have been hypertransfused with blood. To date, we have found three prospective agents that we consider to be potentially useful: 2,3-dihydroxybenzoic acid (2,3-DHB), rhodotorulic acid (RA) and cholylhydroxamic acid (CHA). Each is at a different stage of development. 2,3-DHB is an orally active agent that specifically removes iron and acts as a free radical trapping agent (25) This latter property we hope will decrease the pathology associated with iron overload since the tissue damage is presumably done by the generation of free radicals by iron. A preliminary clinical evaluation revealed that 2,3-DHB caused the excretion of 5-20 mg of iron per day in a series of patients with β-thalassemia major (26). From the preliminary data it appears that 2,3-DHB, like desferrioxamine, will only retard the accumulation of iron since these patients receive approximately 20 mg of iron per day as transfused blood. A longer clinical study is now in progress at New York Hospital-Cornell Medical School.

Rhodotorulic acid (RA) is a hydroxamic acid chelator which is the most active agent that we have found; unfortunately, like desferrioxamine, it has to be administered parenterally. Toxicological studies in animals have recently been completed, and a preliminary clinical study will be initiated in the near future. The hydroxamic acid of cholic acid shows particular promise as an oral iron chelating drug since it is as active orally as desferrioxamine is parenterally (27). Cholylhydroxamic acid (CHA) appears to be transported via the enterohepatic circulation to the liver where it chelates iron. The chelate is then excreted through the bile. It is hoped that a nonabsorbable iron chelator in the diet, like tannic acid or phytates, will then complex with the iron to allow the CHA to be recycled. Further studies of CHA are currently in progress. We hope over the next few years to develop a series of iron chelators which will remove iron from different metabolic pools and allow the patient to be maintained in negative iron balance.

CONCLUSION

In summary, the genetic diseases of man constitute a challenge to the future medical scientists that I hope will be met in the years to come. Perhaps this paper will stimulate some students at this conference to embark on such a career.

ACKNOWLEDGEMENTS

The progress reported above reflects the input of many individuals who have worked unselfishly. I would like to acknowledge each for his hard work.

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CUMULATIVE CONTENTS OF THE PROCEEDINGS

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Pp. 176 ($6.00)

VOLUME 3 (1971)

Pp. 116 ($4.00)