Failures leading to remarkable discoveries:

TNF and its natural inhibitor erythropoietin

The hurtful feelings associated with failing can be devastating especially if the failure occurs after the investment of a considerable effort. The reflection of a lifetime of work in translational medicine has revealed that the study of failures can give birth to new insights that can be explored with important consequences.

BY PROF. DR. ANTHONY CERAMI

Recently, in the defense of the validity of patents that I have been an author of, I reviewed forty years of my discovery work, which consisted of a truck load of file boxes, laboratory note books, scientific papers, correspondence, personal journals and the occasional paper napkin that contained scribbles of a discussion over lunch. The process was overwhelming, but revealed to me one very important truth: many of the biggest discoveries of my career were the results of failure of another research project. Failure strikes a negative tone, but it appeared in my personal history that it was an essential experience on the path to important discoveries. Failures happen all the time, especially in experimental biology, and they can be very disheartening. In this paper, I'm going to embrace the concept that failure has a value, and review with the details of the two failures, which eventually led to discoveries that have turned out to be quite remarkable.

Discovery of tumour necrosis factor (TNF)

In the mid-1970s, I received a grant from Ken Warren of the Rockefeller Foundation as part of the Great Neglected Disease Network to support new research initiatives in the field of parasitology. This was a unique 10-year grant that required us to spend a considerable effort to advance the field. One of the projects I choose to study was trypanosome infections in cattle. Over a 2-year period, we developed several compounds that showed promise as anti-trypanosomal drugs. A single injection could completely cure mice and rats of the parasites. With this information in hand, we traveled to Kenya to evaluate one of these compounds in cattle infected with trypanosomes. One of the main problems with trypanosome infections is that the animals lose rapidly a considerable amount of muscle and fat - literally wasting away until their death (see figure 1).

When we administered our new compound to the infected cows, they proceeded within minutes to go into shock and die of some unknown reason. If we gave the

Professor Tony Cerami has had a remarkable career in translational medicine. Utilizing biochemical insights he has developed a number of therapeutic strategies that have been translated into products of high clinical utility. He is the inventor of the Hemoglobin A1c test that is used by diabetics all over the world. In 1980 he and Masanubu Kawakami wrote the first patent describing the use of antibodies to TNF as an anti-inflammatory therapy. In 1999 he and his colleagues discovered that the cytokine erythropoietin is the natural inhibitor of TNF and subsequently discovered a small peptide that is devoid of hematopoietic properties while retaining tissue protective properties. This peptide is now in clinical development in the Netherlands where he is currently Boerhaave Professor of Medicine at Leiden University. Dr. Cerami is a member of the National Academy of Sciences, the Institute of Medicine and was the former Dean of the Rockefeller University. He has received many international awards and three honorary Doctor degrees. e-mail: acerami@araimpharma.com

Figure 1. *A cow that has been infected with trypanosomes.*

compound to non-infected animals there was no visible effect. These results were, needless to say, quite discouraging and embarrassing.

The joke around the facility was that 'Have you heard that those guys from New York City have developed a great diagnostic to identify trypanosome infected animals – the problem was that the readout was their death.' Although we spent a considerable amount of effort trying to understand the basis of this phenomenon, we never came up with an explanation and eventually abandoned this series of compounds.

One day, when I was in the corral examining the cattle, I developed severe abdominal pains from some unknown but common 'gastrointestinal bug'. I decided to sit in the corral among the cows and wait until the pain stopped. As I sat there I decided to take my mind off the pain by analyzing the events of the past few weeks. The cutting remark that this compound would be useful for identifying infected animals was actually an interesting idea, because in contrast to mice and rats who had vast numbers of organisms, the infected cows had very few parasites in the blood or for that matter in the rest of the body. Then, the question arose in my mind – how could so few parasites cause this tremendous loss of weight and death?

Cachexia and anaemia

A common explanation for cachexia or the wasting associated with infectious diseases or cancer was the usurping of the energy stores of the host for its own growth. In some instances, such as human *Leishmania donovani* infections, the parasite load in the liver and spleen is immense. In the case of the infected cows, it seemed hard to believe that so few organisms could use that much energy. I then remembered speaking with an investigator who was studying trypanosomes in wild antelopes. He found that they developed a level of parasitaemia similar to that seen in cattle, but they did not develop cachexia. It then occurred to me that there was a good possibility that the European cattle were overreacting to the presence of a minute number of parasites and producing a mediator

that was responsible for the wasting. The question was what was this mediator and how could we prevent its action. I thought that if we knew the answers to these questions we would have insight into the pathogenesis of many diseases. Over the next 30 min, I not only laid out the scientific plan that we would follow for the next 10 years but was also relieved of my abdominal pains.

Over the next few years we worked on the isolation of the protein that was made when the body was invaded. We called the molecule cachectin since we believed it was the material responsible for cachexia. In 1981, we submitted an application to the US patent office describing the mediator as a protein, with an apparent molecular weight of 70 000 daltons agents mimicking invasion. A number of the biological activities of the mediator were described as well as the principle of inhibiting its activities with antibodies in a number of human maladies including shock, cachexia, rheumatoid arthritis and other conditions that were the result of elevated levels of this mediator.

Biological activity

Subsequent work showed that cachectin and a molecule called tumour necrosis factor (TNF) were one and the same. This caused a considerable stir in the biotechnology industry since it had been hoped that TNF would be a specific anti-tumour agent that could be administered safely to patients. The activities associated with cachectin, on the other hand, would be detrimental if not lethal to the patient. At one point, I attended an early biotechnology meeting and was accused by one of the participants of wanting to destroy the young biotechnology industry. Implicit in his statement was that our observations on the biological activities of cachectin/TNF were faulty. I assured him that we were confident of all of the biological activities that we described were associated with TNF and that we understood that these activities would be dose-limiting or preclude its use as an anti-tumour agent in patients. I then proposed that the prevention of the activity of TNF with monoclonal antibodies would be a much larger market as so many diseases are associated with elevated cachectin/TNF levels. Unfortunately, except in certain instances, the clinical use of TNF as an anti-tumour agent has not been possible. Clinical studies of TNF confirmed all of the animal studies that we had seen with mouse cachectin. Fortunately for the biotech industry, anti-TNF therapies did succeed and are one of the largest selling biotechnology products sold in the world today.

Over the years, it has become clear that in response to invasion (including bacteria, viruses, parasites, tumours) or tissue damage, there is the local and systemic production of TNF (see figure 2). At the local level, it can induce apoptosis (programmed cell death), production of other cytokines, nitric oxide and other mediators that enlarge the amount of damage and in the process kill the invader.

If the invasion (either real or perceived) is significant, then TNF will spill over into the blood stream and on a systemic level cause fever, anorexia, malaise, a generalized catabolic state, shock and death. Armed with recombinant TNF, it was possible to show all of these biological activities in vivo and in vitro.

Role in inflammatory diseases

The administration of recombinant h-TNF led to a rapid rise in the respiratory rate, due to an increased production of lactic acid, a fall in blood pressure and the subsequent death of the animal. We also believed that cachectin/TNF would play an important role in inflammatory diseases such as rheumatoid arthritis. In collaboration with Jean-Michele Dayer of Switzerland, we were able to show in 1985 that small concentrations of cachectin/TNF could induce human synovial cells to produce PGE2 and collagenase – two mediators that were believed to be involved in the damage of joints in rheumatoid arthritis patients. The chronic administration of TNF to experimental animals can induce all of the changes that are seen in cachexia.

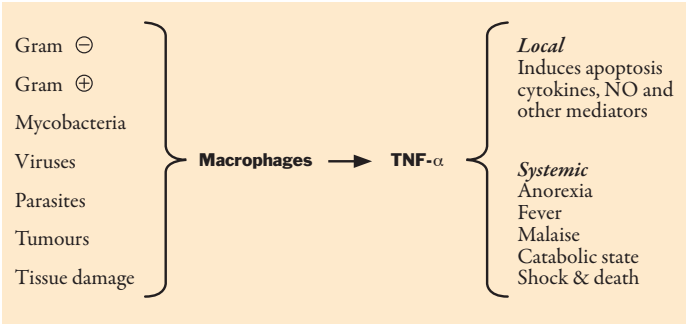

Figure 2. *Effects of TNF in vivo. Many pathogens, tissue damage and tumours have been described to elicit the production of TNF by macrophages. The TNF can have local as well as systemic effects in vivo.*

This was best demonstrated by Alan Oliff and his colleagues who showed that cells engineered to secrete TNF could reproduce all of the sequelae of cachexia (figure 3). Surprisingly, there have been very few clinical studies evaluating monoclonal antibodies to TNF in cancer cachexia. The anti-TNF monoclonal antibodies have proven to be very successful in the treatment of patients with chronic inflammation including Crohn's disease, rheumatoid arthritis and psoriasis. These pioneering clinical studies were carried out by Sander van Deventer, Ravinder Maini and Mark Feldmann utilizing a monoclonal produced by Centocor. An unsung hero in these early endeavors is Jim Woody who, as the head of research at Centocor, promoted and protected these studies.

Figure 3. *The mouse on the top had cells injected into the leg that produced TNF causing cachexia.*

Discovery of erythropoietin (EPO) as an endogenous TNF antagonist

TNF can cause a significant amount of local collateral damage as a result of the body believing that there is an invasion in progress. We concluded that a natural scheme must have evolved to counter the activity of this potent cytokine, otherwise small injuries could lead to devastating damage. For a several year period in the end of the 1980s we began a program to find this mechanism. For several years, we searched for other cytokines made by macrophages that had either pro-inflammatory or anti-inflammatory action. During this period, we identified a number of new pro-inflammatory cytokines, but completely failed in identifying any anti-inflammatory molecules. After a few years of searching, in frustration, the project was abandoned. In 1998, Mike Brines, Carla Hand and I received a grant from Johnson and Johnson to study why EPO administration to cancer patients improved their feeling of well-being. This phenomenon accounted for the widespread use of this cytokine in oncology. As we knew from animal studies that TNF causes malaise that can be demonstrated by a slower rate of learning in a water maze, we thought that in this model, we could evaluate whether EPO could interfere with TNF-activity. As a control, we administered EPO to normal young animals and found to our astonishment that these animals were able to learn the maze faster. After confirming this experiment many times, we hypothesized that as EPO had the

opposite activity of TNF in this model, perhaps it could counteract TNF in other situations. In a series of experiments, we were able to show that EPO indeed had the opposite profile to that seen with TNF, e.g. decreasing apoptosis and inflammation while improving the rate of healing and regeneration of tissue after damage.

The administration of EPO to experimental animals, having been subjected to many kinds of injury, has been found to reduce the amount of damage greatly. These include stroke, brain and spinal cord trauma, ischaemia reperfusion models of the retina, heart, kidney and bowel, to name a few (figure 4).

Interestingly, a clinical study of EPO administration once a week for 4 weeks to trauma patients admitted to the ICU showed a 50% decrease in death rate at 28 days compared with the saline controls. Despite this significant decrease in mortality, the patients receiving EPO also had a 40% increased risk of thrombosis or blood clotting. This side effect of EPO is well known and is one of the reasons why EPO has a 'black label' warning by the FDA.

It was readily apparent that the potential of EPO as a tissue-protective molecule was greatly diminished by this activity. The question was whether we could engineer the molecule to remove this activitiy. In pharmacology, this can successfully be achieved in situations where there are two different receptors. Early in our studies it became clear that the amounts of EPO that we needed for tissue protection were significantly higher than that needed for making red cells, suggesting that the tissue-protective receptor had a lower affinity. We were subsequently able to identify the tissue protective receptor that EPO bound to. Some of these are pieces of EPO, while one is a linear peptide of 11 amino acids that reflect the amino acids in space along a helix that, we believe, interacts with the tissue-protective receptor. Further work led us to the identification of an eleven amino acid peptide, ARA290 that is not a piece of EPO, but represents the amino acids in space that interact with the new receptor. In a number of animal models ARA290 is as active as the parent molecule EPO but without the safety issues associated with EPO.[1]

Pre-clinical animal studies and clinical studies in human volunteers have not revealed any untoward effects. Phase 2 clinical trials in patients with painful neuropathy caused by sarcoidosis and diabetes are underway. Whether this peptide will be as effective in clinical medicine as anti-TNF therapies will have to await the results of these trials. Although I believe that examination of failure can lead to new discoveries, I hope that these clinical trials will be successful as I would not like to have to worry about another failure.

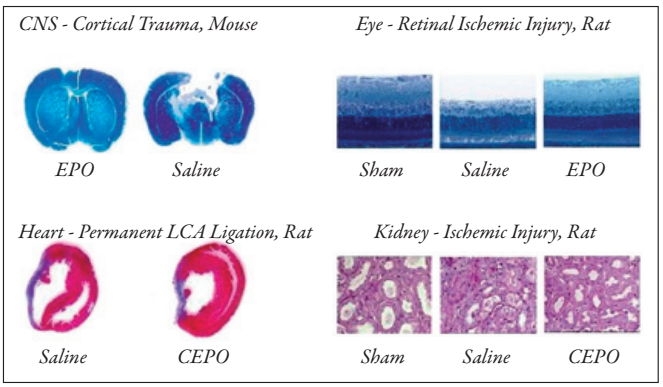

Figure 4. *Tissue-protective molecules (erythropoietin and carbamylated-erythropoietin, a nonerythropoietic molecule) can limit injury in many tissues. In this figure are shown the protective effects from brain trauma, retinal ischaemia, myocardial infarction and kidney ischaemia reperfusion injury are shown. Sham refers to the fact that animals were operated on but did not experience the ischemia/reperfusion procedure. In effect, they are normal animals.*

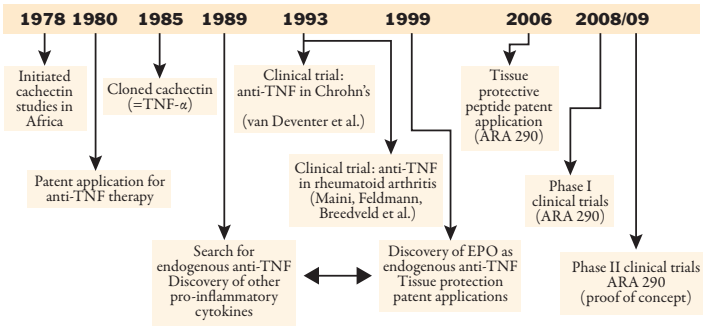

Figure 5. *Developmental timeline of important points in the discovery and development of TNF and its natural inhibitor, erythropoietin.*

Final reflections

Two failures have led to remarkable discoveries. The first led to the discovery of TNF as an important mediator of inflammation that can, if unchecked, cause severe damage in mammals. The second is the identification of erythropoietin as the natural inhibitor of the production and biological activity of TNF. The other observation I made as I reviewed my life's work in translational medicine was that considerable patience was needed. Figure 5 shows a time line for the development of the programs I have discussed above. In both instances, there was about a 10-year period between the basic discovery until clinical studies could be carried out to determine whether the approach would be successful. During this period, there were many discouraging moments of conflict and uncertainty, but in looking back, I am glad that I chose this difficult path. To be able to discover diagnostics or therapies that can relieve pain and suffering is very rewarding. I hope that young people reading this paper will be encouraged to choose to follow this long and arduous path and will help give students the courage to persist in looking for the insights that are the by-products of failure, and to understand the long time lines in the path of discoveries.

Reference

1. Cerami T. The value of failure: the discovery of TNF and its natural inhibitor erythropoietin. J Intern Med: 2010; 269: 8-15. *A more detailed scientific presentation of this work and all relevant references can be found in this paper.*

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