The value of failure: the discovery of TNF and its natural inhibitor erythropoietin

A. Cerami
Leiden University Medical Center, Leiden, The Netherlands


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. This article discusses the analysis of two failures that 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. I hope that this paper 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.

Keywords: biological therapy, erythropoietin, immunomodulation, inflammation, tissue protection, TNF.

Introduction

Recently, in the defence 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. I hope that students reading this paper will use the lessons of these examples to help alleviate the pain of failing and give them courage.

The discovery of 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. This came about because of the interesting work that had been done at the beginning of the 20th century by the famous pharmacologist, Paul Ehrlich. He discovered the first anti-trypanosomal drug for the treatment of cattle in Africa. Of historical interest, when the spirochete causing syphilis was first discovered, Ehrlich thought that this organism was a small trypanosome and began to try the compounds that he had developed for trypanosomes on this spirochete. We now know that these organisms are not related, but it did lead to the development of the magic bullet 606, a highly potent drug for the treatment of syphilis.

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 travelled 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. When we administered our new
A. Cerami | Key Symposium: The discovery of TNF and its natural inhibitor erythropoietin

compound to the infected cows, they proceeded within minutes to go into shock and die of some unknown reason. If we gave the 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 analysing 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?

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.

Upon returning to New York, Carol Rouzer, a graduate student, began to study rabbits infected with trypanosomes that like the cow had a very low parasitaemia, but severe wasting. We began by studying the anaemia associated with the cachexia of this infection.

We found that one of the main reasons for the anaemia was the inability to make new red cells. She then made the observation, that during the course of the infection, the serum of the animals became extremely lipemic [1]. Further studies revealed that this was due to the loss of the enzyme lipoprotein lipase (LPL) (Fig. 1). It was clear that study of the loss of this enzyme in response to invasion would be a good biomarker to identify and isolate the mediator.

The project was then turned over to Masanobu Kawakami, a dedicated and talented post-doctoral fellow. We decided to switch from trypanosome-infected rabbits to mice that were challenged with endotoxin or lipopolysaccharide (LPS). It had been known that when some strains of mice were injected with endotoxin, they developed severe organ damage that led to death, whereas, other strains of mice were resistant. We performed a very simple experiment in which we showed that the administration of LPS to the sensitive mouse led to a decrease in LPL, whereas the administration of LPS to the resistant mouse did...
not. We were then able to show that if we took sera from the sensitive mouse that had been injected with LPS 60 min previously and gave it to a resistant mouse, there was a suppression of LPL—signalling the presence of a mediator (Fig. 2). We continued to simplify our animal models by showing that macrophages from sensitive mice when incubated with LPS also produced the mediator that could suppress LPL in the resistant mice [2].

Subsequently, we were able to simplify our models even more by developing an in vitro assay with Phil Pekala and Dan Lane of Johns Hopkins utilizing 3T3/L-1 cells that had been induced to form fat cells. The addition of the mediator to these fat cells led to a specific turning off of LPL and other enzymes responsible for fatty acid synthesis and uptake [3, 4]. This LPL assay formed the basis for our isolation of the mediator. In a series of papers, we showed that the supernatants of macrophages that had been exposed to LPS or other molecules from infective organisms had the ability to induce a mediator with many activities that had been associated with cachexia and anaemia [5–7]. Although we believed that there was a single mediator, it was only after we had recombinant cachectin/TNF that we could say conclusively that there was only one mediator that had these many diverse biological effects.

In 1981, we submitted an application to the US patent office [8] describing the mediator as a protein, with an apparent molecular weight of 70 000 daltons as measured by gel filtration chromatography, that was produced by macrophages in response to various agents mimicking invasion. A number of the biological activities of the mediator were described as well as the principle of inhibiting its activities with poly- and monoclonal 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. At this point, we named the mediator cachectin because many of its biological activities were associated with the phenomena of cachexia.

At this point in time, micro-sequencing of small quantities of protein was just beginning. Most batches of our purified material were lost attempting to obtain the amino terminal sequence. In desperation, I reached out to Dr. Y. Pan of Roche who had just obtained the latest sequencing apparatus. On his first attempt, he was able to obtain the amino-terminal sequence of mouse cachectin that had been isolated by Bruce Beutler, a post-doctoral fellow [9]. Examination of the available databases for other proteins did not reveal any similarity in sequence. A few days later, Bruce noticed that the sequence we had obtained for mouse cachectin was very similar to that of the recently cloned protein, human TNF. This came as a big surprise to us because several years before, we had exchanged material with Lloyd Old’s group at Sloan-Kettering to see if TNF had LPL suppressive activities or cachectin had anti-tumour activity. Both assays were negative. We were subsequently able to show that mouse cachectin and mouse TNF were identical [10]. Why neither laboratory was able to find the other activity remains a mystery.

The fact that cachectin and TNF were in fact the same caused a considerable stir in the biotechnology field. It had been hoped that TNF would be a specific anti-tumour agent that could be administered safely to patients [11]. 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 [12]. Clinical studies of TNF confirmed all of the animal studies that we had seen with mouse cachectin [13, 14]. 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 (Fig. 3). At the local level, it can induce apoptosis, production of other cytokines, nitric oxide and other mediators that enlarge the amount of damage and in the process kill the invader [15]. 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 [16, 17].

Armed with recombinant TNF, it was possible to show all of these biological activities in vivo and in vitro. 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 (Fig. 4). We also believed that cachectin/TNF would play an important role in inflammatory diseases such as rheumatoid arthritis. In collaboration with Jean-Michele Dayer, we were able to show in 1985 that small concentrations of cachectin/TNF could induce human synovial cells to produce PGE2 and collagenase [18] – two mediators that were believed to be involved in the damage of joints in rheumatoid arthritis patients.

Utilizing recombinant human-cachectin/TNF, we were able to produce specific monoclonal antibodies. One of these antibodies was able to cross-react and neutralize the bioactivity of baboon TNF. We were able to evaluate its potential in preventing septic shock in animals that were given a lethal amount of live Escherichia coli (Fig. 5) [19]. When the antibody was administered 4 h before the infusion of the bacteria, there was a complete prevention of the pathological sequelae and death that occurred in the saline treated animals. All of the control animals died within the first 7 h. If the animals were given the antibody at the time of the infusion of bacteria, the animals also died. The timing of the administration of the antibody was obviously critical and probably accounts for the lack of clear-cut clinical efficacy in human trials of anti-TNF antibodies in septic shock.

The chronic administration of TNF to experimental animals can induce all of the changes that are seen in cachexia. This was best demonstrated by Alan Oliff and his colleagues who showed that CHO cells secreting TNF could reproduce all of the sequelae of cachexia [20]. The administration of anti-TNF antibodies to mice bearing tumours revealed a significant alleviation of the sequelae of cachexia [21]. 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 [22], Ravinder Maini and Mark Feldmann [23] utilizing a monoclonal produced by Centocor. An
The unsung hero in these early endeavours is Jim Woody who, as the head of research at Centocor, promoted and protected these studies.

The 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 programme 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 [24–27]. 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 accounts 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 intraperitoneally 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. Another interesting observation we have found is that EPO and TNF can regulate the production and the activity of the other. Thus, elevated TNF levels can suppress the production of EPO that accounts, in part, for the anaemia associated with cachexia. The mistake we made in our earlier programme to find the mechanism that countered TNF-associated damage was to assume that the inhibitor of TNF would be produced by macrophages. It never occurred to us that the surrounding cells would be the source. The signal for local production of EPO is the activation of HIF 1 by hypoxia that is the caused by TNF impairing blood flow.

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 [28–31] (Fig. 6). Interestingly, a clinical study of EPO administration (40 000 units) 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 [32]. Despite this significant decrease in mortality, the patients receiving EPO also had a 40% increased risk of thrombosis. 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 these other activities. The question was whether we could engineer the molecule to remove these other activities. 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 erythropoiesis, suggesting a tissue-protective receptor with a lower affinity. We then studied two EPO derivatives that were previously reported to not be erythropoietic in vivo. Asialo-EPO can sustain erythropoiesis in vitro, but is completely inactive in vivo because of its short half-life of a few minutes. Carbamylated-erythropoietin (CEPO which is made by the reaction of cyanate with the amino groups of EPO) is not erythropoietic in vitro or in vivo. When these compounds were evaluated in vivo, both were found to retain completely the tissue-protective activities [33, 34].

Utilizing a CEPO-column, we were able to isolate the tissue-protective receptor [35]. It was found to be composed of the EPO-receptor disulfide linked to CD131, commonly known as the beta-common receptor because of its participation with other specific alpha chains for the cytokines IL3, GM-CSF and IL5 (Fig. 7). In the early history of EPO, this receptor had been isolated and was found to be phosphorylated after exposure to EPO [36]. Subsequent studies showed that the homodimer of EPO-R was the haematopoietic receptor [37]. In addition, the beta-common knock out mice for the beta-common gene did not have any haematopoietic deficits [38]. When these mice were damaged in vivo or their cells in vitro, EPO was no longer able to protect against collateral damage. The tissue protective receptor has a lower binding constant, 16 nM, compared to 0.2 nM for the homodimer of EPO-R. Another important difference is that homodimer requires low constant levels to maintain haematopoietic effects, whereas the tissue-protective receptor requires high, but infrequent levels.

As the x-ray structure is known, we have been able to design EPO molecules that have lost their haematopoietic activities by substituting critical amino acids of EPO needed for binding to the EPO-R homodimer. There are a number of problems associated with the production of large protein molecules by mammalian cells. The first is the cost of the regulatory barrier (estimated to be $20–25 million) that needs to be overcome to do even proof of concept clinical studies and the second is the cost of goods when the product is eventually sold to patients. These were challenges that we could not meet.

The fact that asialo-EPO, which has an extremely short half-life in vivo, has all of the tissue-protective activity of EPO, encouraged us to evaluate peptides as tissue-protective molecules [39]. We have identified a number of peptides that are tissue protective in vivo. 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. This peptide, ARA290 that is not a piece of EPO, is as effective at tissue protection on a molar basis in a number of animal models as the parent molecule EPO. Thus, we were able to obtain a chemically synthesized tissue-protective molecule, reduce the molecular weight by nearly thirty fold and remove the biological activities (erythropoiesis, producing hyperactive platelets, promoting coagulation and increasing blood pressure) that were safety issues associated with EPO.

Pre-clinical animal studies and clinical studies in human volunteers have not revealed any untoward effects. Phase 2 clinical trials in patients with critical limb ischaemia, painful diabetic neuropathy and retinopathy and rheumatoid arthritis are underway or in the planning stages. 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.

Final reflections

The other observation I made as I reviewed my life’s work in translational medicine was that considerable patience was needed. Figure 8 shows a time line for the development of the programmes 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.

Acknowledgements

I thank Ann Dunne, Ed Gray and Ulf Andersson for helpful discussions.

Conflict of interest statement

Anthony Cerami is a share holder of Araim Pharmaceuticals that owns the patent on peptides that mimic the tissue protective activities of erythropoietin.

References

A. Cerami  

Key Symposium: The discovery of TNF and its natural inhibitor erythropoietin


Correspondence: Anthony Cerami, Leiden University Medical Center, Leiden, The Netherlands and Arahim Pharmaceuticals, Inc, 712 Kitchawan Rd. Ossining, NY 10562 (Town of Yorktown). (fax: +914-762-7292; e-mail: acerami@araimpharma.com).