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PERSPECTIVE

Tumor Necrosis Factor (TNF)

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Spontaneous regressions of cancer, rare events but repeatedly recorded, have led generations of investigators to seek explanations for their occurrence and therapeutic maneuvers to increase their frequency. When a number of patients undergoing cancer regression in the late 1800's were found to have concurrent bacterial infections, F. Fehleisen in Germany and William B. Coley in the United States, as well as a small group of other physicians, attempted to induce infections in patients with far advanced cancer. Although antitumor responses were seen, some dramatic, it was difficult to infect most patients, and, when an infection did occur, there was no way to control its severity. Coley, therefore, turned in 1893 to the use of killed bacteria, and the mixture of *Streptococcus pyogenes* and *Serratia marcescens* that he and others used to treat cancer came to be known as Coley's toxins. Coley's work was well known at the time, and in 1934 the American Medical Association stated that Coley's toxins were the only known systemic therapy for cancer. However, with advances in radiotherapy and, subsequently, chemotherapy, clinical interest in toxin therapy diminished, even becoming controversial in certain quarters. Coley's results would have been lost had not his daughter, Helen Coley Nauts, collected and analyzed the records of her father and other physicians from this country and abroad (*1*).

In contrast to the eclipse of clinical interest in toxin therapy, laboratory studies of microbial products as antitumor agents have had a long and uninterrupted history. A wide range of microorganisms have been examined, from bacteria, yeast and other fungi to plasmodia and trypanosomes, but most attention has been focused on three groups of organisms: Gram-negative bacteria, mycobacteria such as Bacillus Calmette-Guérin (BCG), and corynebacteria such as *Corynebacterium parvum*. One of the most dramatic and reproducible phenomena in experimental tumor biology is the hemorrhagic necrosis of certain mouse tumors that can be seen shortly after the injection of filtrates from cultures of Gram-negative bacteria. Murray Shear and his colleagues at the National Cancer Institute identified the active principle as a polysaccharide (*2*), and subsequent work showed that this component, also known as endotoxin or bacterial pyrogen, is a lipopolysaccharide (LPS) and a major constituent of the cell wall of Gram-negative bacteria.

Clinical applications of Shear's findings were limited because LPS was considered to be too toxic in humans. BCG and *C. parvum*, however, were subjected to extensive tests in cancer patients, with generally disappointing results. The clinical use of BCG and *C. parvum* was based on a large series of animal studies, starting with the demonstration by Baruj Benacerraf and myself that BCG-infected mice showed heightened resistance to challenge with transplantable tumors (*3*). It was generally considered that the action of LPS and BCG was indirect and mediated by the host. In the case of LPS, Glenn Algire of the National Cancer Institute suggested that tumor hemorrhagic necrosis was secondary to LPS-induced hypotension and collapse of tumor vasculature. The systemic antitumor effect of BCG and agents with similar activity was thought to be due to a general augmentation of immunological reactivity, since BCG-infected mice were more resistant to bacterial and viral challenge, rejected incompatible skin grafts more rapidly, and produced higher titers of serum antibody. For many years there has been speculation that macrophages play a key role in the antitumor activity of microbial products. Both LPS and BCG have profound effects on macrophages, activating them to become more phagocytic and more bactericidal. In addition, activated macrophages have the capacity to inhibit or destroy cancer cells in vitro through a variety of mechanisms, including the production of active oxygen intermediates (*4*).

The discovery of tumor necrosis factor (TNF) provided a clue as to how these diverse reactions to microbial products might be linked. It was during an investigation of the antitumor activity of normal serum, particularly the leukemia inhibitory activity of serum, that we found TNF (*5*). In attempts to modify the level of antitumor factors in the blood, we tested serum from mice injected with BCG, LPS, or both agents together. The serum of BCG-infected mice injected with LPS (but not serum from mice injected with either agent alone) caused hemorrhagic necrosis of an LPS-sensi-

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tive mouse sarcoma and complete regression of tumors in a proportion of treated mice. Our first thought was that residual LPS in the serum accounted for its activity, but this seemed unlikely because the amount of LPS injected in the BCG-infected mice was far lower than required to induce hemorrhagic necrosis on its own. In addition, serum from BCG-LPS injected mice was strongly cytotoxic for mouse L cells, a transformed fibroblast cell line, whereas the serum of mice injected with BCG or endotoxin alone was not active against L cells, nor was there any direct cytotoxic action when BCG and endotoxin were added to cultures of L cells. It soon became clear that two events in proper sequence were necessary for production of the tumor necrotizing and cytotoxic factors. The first was a priming event that caused activation and proliferation of macrophages and was associated with expansion of reticuloendothelial elements in liver and spleen; for priming, *C. parvum* and zymosan (yeast cell walls) functioned as well as BCG. The second event (elicitation) was required for appearance of the factor in the blood, and LPS turned out to be unique as an eliciting agent. Using these principles, one could obtain sera with similar properties from rats and rabbits.

Although these findings were made in 1971, we delayed their publication until 1975 because of the need to convince ourselves that residual exogenous LPS or endogenous LPS released from the intestine was not involved in serum-induced hemorrhagic necrosis. There were many claims in the literature for new factors mediating LPS-like reactions that turned out to be due to contamination with LPS or LPS-containing bacteria, and we did not want our work to suffer a similar fate. However, a series of studies involving LPS assays, LPS tolerance, and LPS inhibitors provided the assurance we needed that the serum necrotizing factor was not LPS. The other key issue was whether the necrotizing factor and the cytotoxic factor were one and the same, and our purification efforts over the years, first with Saul Green (*6*) and then with Katsuyuki Haranaka, led to the conclusion that the two activities could not be separated. Both activities in sera can be found in fractions of high (150,000), intermediate (40,000), and low (18,000) molecular weight. Haranaka's purification scheme of rabbit and mouse serum TNF leads to a protein with a molecular weight of 40,000; this protein dissociates into components of 18,000 molecular weight on exposure to sodium dodecyl sulfate (*7*).

After the initial publications on TNF, a number of investigators, particularly N. Matthews in Wales, and G. Gifford, S. E. Mergenhagen, and P. Cuatrecacas in this country, turned to the study of serum TNF. Because the in vitro cytotoxic assay was easier and required less material than the in vivo tumor necrosis assay, much of the work on TNF focused on its cytotoxic activity. Because L cells, the standard TNF assay cell, can be killed by any number of substances, a specificity control that distinguishes TNF killing from other cytotoxic factors was required (particularly when actinomycin was used to potentiate TNF action). TNF-resistant lines of L cells can be derived from sensitive populations by repeated growth in TNF-containing media, and tests on paired TNF-sensitive and TNF-resistant L cells have served us well in identifying TNF and TNF-like molecules in animals and humans. For example, we and others have shown that mouse macrophages and cloned lines of histiocytomas produce a factor that is cytotoxic for L cells, and that levels of this factor can be increased by LPS. The fact that TNF-resistant L cells were not killed by the macrophage factor indicated its relation to TNF and added to the considerable indirect evidence that macrophages were the primary source of serum TNF. Another characteristic of mouse TNF that became apparent in the study of its in vitro cytotoxic activity was a lack of species specificity (*8*). In a survey of 62 cultured cell lines from a wide range of human cancers, Akihiro Yamamoto found that mouse TNF had a cytotoxic effect on 19 lines, a cytostatic effect on 21, and no effect on 22. Cultured normal human cells, including fibroblasts, kidney epithelium, and melanocytes, were not inhibited by TNF.

We then began to search for a similar substance in humans. A large number of human cell lines of hematopoietic origin were screened, and one lymphoblastoid cell line, designated LuKII, produced particularly high levels of a factor that was cytotoxic for TNF-sensitive (but not TNF-resistant) L cells, caused hemorrhagic necrosis of TNF-sensitive mouse sarcomas, and showed the same pattern of reactivity as mouse TNF on the panel of human cell lines (*9*). Since we knew from the work of William Stewart that LuKII cells also made interferon, we set out to prove that interferon was not involved in the reactions ascribed to TNF. The answer was clear: purification of the LuKII supernatant resulted in fractions with TNF activity but no interferon activity; human α-, β-, and γ-interferons had no activity in the standard TNF assays; and the inhibitory activity of interferon on the human cancer cell panel was distinguishable from TNF. As a consequence of these studies, however, we did uncover another important feature of TNF: synergistic action with interferon (*9*). Mouse and human TNF and interferon are strongly synergistic in their in vitro cytotoxic effects.

It was at this point in the TNF story that the gene coding for human TNF was cloned and expressed (*10–14*). The field was instantly transformed, and TNF, which had been the concern of a relatively small group of investigators, suddenly found itself the focus of intense scientific, medical, and commercial scrutiny, with expectations about its potential as an antitumor agent also rising dramatically. Cloning is an important rite of passage for biological factors such as TNF, and there is a growing sense that a factor has to be cloned before it is taken very seriously. (To paraphrase Descartes: "It's been cloned, therefore it exists.") Two general strategies were chosen for TNF cloning. The strategy taken by the Japanese groups started with purified rabbit serum TNF and had direct links with our original work on TNF, because K. Haranaka introduced methods of TNF production, purification, and assay to these groups when he returned to Japan in 1980 after his work in my laboratory. Genentech and Cetus, on the other hand, chose TNF produced by the HL-60 cell line as the source of material for microsequencing and the development of probes. A consistent picture has emerged from the study of cloned TNF, which appears to be encoded by a single copy gene. Four exons code for a precursor product of 233 amino acids and a mature product of 157 amino acids after an unusually long leader sequence has been removed. The molecular weight of the human recombinant TNF product is 45,000 by gel filtration and 17,000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and these values are in excellent agreement with conclusions reached in the study of mouse and rabbit serum TNF. Mouse TNF has also recently been cloned, and mouse and human TNF show approximately 80 percent homology at the amino acid level (*15, 16*).

In all ways but one, recombinant TNF behaves exactly as one would have predicted from work with nonrecombinant TNF, that is, it causes hemorrhagic necrosis of mouse tumors, shows the same pattern of cytotoxic reactivity for mouse and human cells, and has synergistic cytotoxic activity with interferon. What we had not seen and was therefore unex-

pected was lethality due to TNF. Although mice injected with nonrecombinant sources of TNF show some ruffling of fur and weight loss, we had never seen deaths attributable to TNF alone. It is not clear at this point whether the toxicity of recombinant TNF is simply a matter of dosage, or whether other factors are involved, such as structural differences between recombinant and nonrecombinant TNF or the presence of toxic impurities in the cloned preparations. From our initial work on TNF, we postulated that TNF is a mediator molecule evoked by LPS and involved in LPS action. From this point of view, toxicity due to TNF would not be surprising if TNF mediates this property of LPS as well. The increased sensitivity of tumor-bearing mice to LPS is also seen with TNF, and the fever and hypotension occurring in the first patients treated with cloned TNF in Japan are also reminiscent of LPS reactions in humans. What is needed now is a tally of how many LPS reactions can be reproduced by TNF or be inhibited by antibody or other blockers of TNF action.

Recent evidence indicates that TNF belongs to a family of molecules having similar biological activities and varying degrees of structural relatedness, reminiscent of findings with interferon. The gene coding for a cytotoxic factor called lymphotoxin has been cloned by a group at Genentech, and sequence comparisons show that TNF and lymphotoxin are clearly related (*17*). Lymphotoxin is produced by mitogen-stimulated lymphocytes and has been thought to play a role in lymphocyte-mediated killing (*18–20*). Cloned lymphotoxin is cytotoxic for L cells and causes hemorrhagic necrosis in the standard TNF assay. Another factor, called cachectin, has strong sequence homology with TNF, and may in fact be identical to TNF (*21*). Cachectin is a product of macrophages and is assayed by its ability to suppress the activity of lipoprotein lipase in cultured adipocytes (*22*). Cerami and his colleagues have postulated that the hypertriglyceridemia and wasting associated with certain parasitic diseases is due to the production of cachectin (*22*). How many distinct members of the TNF family will there be? The factor produced by the LuKII human lymphoblast cell line described above has been purified (*23*) but not sequenced, so its identity is not established. Haranaka has recently cultured a cell line from a patient with monocytic leukemia that produces a TNF-like factor, but this factor appears to be only distantly related to human TNF and lymphotoxin. Another question is whether TNF production is restricted to cells of hematopoietic origin or do other cell types produce TNF-like molecules? One way to approach these questions is to screen the large number of human cancer cell lines that are now available for factors with TNF activity.

What are the functions of a molecule like TNF? It seems unlikely that evolution had cancer in mind when fashioning TNF, and a role in infectious diseases seems more likely. Tests on a range of bacteria and fungi have not resulted in any evidence for direct TNF sensitivity. It has been suggested that TNF has inhibitory activity for malarial organisms, but James Jensen and his colleagues at Michigan State University did not find any inhibition of *Plasmodium falciparum* in vitro in assays with cloned TNF. Since TNF has striking effects on polymorphonuclear function (*24*), it may be one of the regulatory signals that control cellular reactions during infection. Another possibility raised by the work with cachectin (*22*) is a role in mobilizing the energy reserves required by the infected host. Tumors may also elicit such a response, either by producing a factor that induces TNF release by macrophages or by producing TNF itself, and there is considerable interest in the possibility that TNF may be involved in some of the metabolic disturbances of cancer patients, including cachexia.

Finally, what are the prospects for human cancer therapy with TNF? The response of subcutaneous transplants of mouse and human tumors to TNF is certainly dramatic (*5, 25*), probably involving a direct action on tumor vasculature and a direct effect on tumor cells. However, primary mammary tumors of mice show no reactions to TNF, even after intratumoral injection, and little work has been done on TNF-sensitive tumors growing in sites other than subcutaneously. The synergistic action of TNF and interferon provides strong rationale for their combined use in the clinic, and the potentiation of TNF cytotoxicity by agents such as actinomycin, mitomycin C, and vinblastine suggests other types of combination therapy. An important note for clinical trials has to do with the character of TNF-induced resistance. TNF-sensitive cells, such as L cells, can be made resistant, but this resistance at its early stage is reversible, since such cells grown in medium without TNF revert to TNF sensitivity. Much effort is now being directed at defining the TNF receptor, and it is now clear that high-affinity TNF receptors exist on the cell surface (*26, 27*). Although the initial hope was that the presence of TNF receptors might be correlated with TNF sensitivity and provide a predictive test for tumor response in individual patients, we and others have detected TNF receptors on a range of cell types, both TNF-sensitive and -resistant. Thus, the basis for the selective toxicity of TNF needs to be sought elsewhere.

As clinical trials with TNF are started in this country and abroad, expectations are high, surely too high based on our current knowledge and the limited types of animal models that have been tested. Nevertheless, factors such as TNF, interferons, interleukin-2, and monoclonal antibodies are quickly ushering in a new era in cancer biology and therapy. The story of TNF is another vivid demonstration of the rapid rate that laboratory observations can now be translated into clinical realities and the profound influence that the biotechnology industry has on this process.

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