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INTERLEUKIN 2 IMMUNOSTIMULATION

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INTRODUCTION

In the history of medicine, the stimulation of host defenses has been an elusive goal that has become attainable only within the past decade. By comparison, immunosuppression has been a part of the therapeutic armamentarium for the latter half of this century. Immuno-stimulation has lagged so far behind immunosuppression as a result of two fundamental difficulties. First, there was inadequate knowledge as to the cellular composition of the immune system, and as to how the system is regulated via immunological molecules. Second, as a consequence of this lack of knowledge, there have been no suitable therapeutic agents that could be used to make the system function better. As a result, early attempts at immuno-stimulation yielded severe toxicity. A century ago, living bacteria and then bacterial extracts were used in attempts to promote inflammation for the treatment of cancer. However, the treatments were so toxic that the therapy was often worse than the disease, and such attempts were largely abandoned with the advent of radiotherapy in the 1920s and then chemotherapy in the 1940s. However, we have come a long way since the use of bacterial toxins, and non-toxic immuno-stimulation is finally within our grasp. This chapter is devoted to a detailed examination of the therapeutic use of the first hormone of the immune system, interleukin 2 (IL2).

HISTORCAL CONSIDERATIONS

The Humoral & Cellular Schools

To understand why immuno-stimulatory therapy lagged so far behind immuno-suppressive therapy, and to understand why immuno-stimulatory therapy can be so toxic, it is necessary to study the history of immunology in the past century. At the turn of the twentieth century, the science of immunology was just beginning and two schools of thought developed. With the discovery of antibodies by Von Behring and Kitasato in 1890(1), the humoralist school developed at the Robert Koch Institute in Berlin, and the notion of using serotherapy to treat infectious diseases evolved. At the time, great excitement was generated, in that the transfer of sera from immunized animals to infected humans could be shown to have marked efficacy. However, the phenomenon of “serum sickness”, a systemic inflammation due to the immune response to the heterologous serum components, limited the use of serotherapy. Therefore, when chemotherapeutics and antibiotics were developed in the 1930s and 1940s, serotherapy gradually became forgotten.

The second immunological school in the early part of the twentieth century formed around Eli Metchnikoff, a Russian biologist who first described phagocytosis.

Metchnikoff recognized that the host defenses must have a cellular basis, and as Chef de Service at the Pasteur Institute, Metchnikoff's studies forwarded the concept that the cellular immune response was primary, and responsible for the humoral immune response. Unfortunately, Metchnikoff was several generations ahead of his time. Although he recognized the phenomenon of phagocytosis and identified two distinct phagocytic cells, i.e. macrophages and microphages (polymorphonuclear leukocytes), the composition and cellular origin of antibodies still remained unknown. Metchnikov postulated that the phagocytes themselves were responsible for elaborating the "fixatives" detectable within plasma (2). The role of lymphocytes in the immune system had not yet been recognized. Moreover, the promise of attenuated microorganisms introduced by Pasteur for use as vaccines was not realized (3, 4). It proved difficult, if not impossible to attenuate the virulence of most pathogens. Accordingly, during the first half of the twentieth century the science of immunology became preoccupied with studies focused on the identification and characterization of the structures and functions of antibodies, and the science of microbiology focused on studies of the characteristics of individual microbes. Any thoughts regarding the cellular origin of the antibodies, serotherapy, vaccines and immunotherapy were largely abandoned.

The Antibody Puzzle

As the structures of antibody molecules gradually became determined after several decades of research (5, 6), by the 1950s immunologists started to ponder how the tremendous diversity of antigen recognition could be created. Neils Jerne proposed that the diversity of recognition was inherent in the system, and that upon introduction of antigen, antibodies were selected naturally, as in a Darwinian sense, from the population that already existed (7). However, the underlying cellular basis for such diversity remained problematic until Burnet proposed the clonal selection hypothesis, which held that a single cell was responsible for the production of only one specific antibody (8).

Proceeding beyond theory proved difficult, in that the cells responsible for the formation of antibodies remained obscure. Like Metchnikoff 50 years earlier, Jerne postulated that antigen-antibody complexes phagocytosed by the antibody forming cells was the stimulus for the production of additional antibodies (7). Therefore, even then, it was not clear how the antigen-antibody reaction could lead to the production of larger amounts of the specific antibody. Various theories were proposed to account for this phenomenon, but there appeared to be no way of testing the hypotheses forwarded. Sir McFarland Burnet succinctly stated the problem confronting immunology in 1959 (9):

"Until such methods of handling pure clones of cells in vitro are available, a choice between the clonal selection hypothesis of antibody formation and other alternatives can only be on the basis of convenience and their heuristic value in stimulating new experiments."

Cellular Immunology

Consequently, investigators focused on identifying and studying the cells comprising the immune system for the next two decades, thereby creating the science of cellular immunology. Peter Nowell pioneered this new science by reporting in 1960 that lymphocytes are capable of proliferating in response to stimulation by mitogens (10), and his discovery was soon followed by the demonstration that specific antigens could also activate the proliferative expansion of antigen-selected cells. Prior to this discovery it had been thought that lymphocytes were end-stage cells, incapable of self-renewal, and they were not thought to be the source of the antibody forming plasma cells. This understanding allowed Burnet's clonal selection hypothesis to be modified to include the proliferative expansion of the clones of antigen-specific cells after they had been selected. Then, in 1963 Jerne and Al Nordin introduced an assay capable of detecting individual antibody forming cells, and the selection of antigen-specific cells, as well as their proliferative expansion was visualized and quantified for the first time (11).

Subsequently, in 1967 Richard Dutton and Robert Mishell improved upon Jerne's assay for antibody forming cells by performing the entire immune response in vitro (12). This development was critical, in that it permitted a reductionist approach to both the activation phase, as well as the effector phase of the immune response for the first time. Armed with these new cellular assays, it became recognized that there were at least three distinct types of cells comprising the immune system, i.e. B cells, T cells, and macrophages. Although plasma cells were found to be the actual antibody forming cells, it was not shown that B cells are the precursors of plasma cells until 1970, when B cells were found to express immunoglobulin molecules on the cell surface (13, 14). However, by 1970 it was realized that antibody formation by B cells/plasma cells was dependent upon help derived from T cells and macrophages, although the mechanisms responsible for this help remained obscure (15).

The interdependence of the cells in generating an antibody response was further confounded by the observations of Baruch Benacerraf and co-workers, who reported that antibody production was genetically determined, and that the cellular immune response was somehow related to this genetic linkage (16, 17). Subsequently, Hugh McDevitt and co-workers reported that the magnitude of antibody production reactive with synthetic peptide antigens was dependent upon the genes encoded by the Major Histocompatability Complex (MHC) (18, 19). Together, Benacerraf and McDevitt demonstrated that this Immune response (Ir) gene effect depended on T cell recognition of antigen (20).

Throughout the 1970s it remained a mystery as to how B cells, T cells and macrophages cooperated to generate the production of antibodies. However, between 1965 and 1975 there were a growing number of reports of activities in the culture medium of proliferating lymphocytes that promoted or augmented the function of in vitro immune responses. Depending on the assays used to detect the activities, they were shown to augment either the generation of antibody forming cells, so-called T cell Replacing Factors (TRF) (21), or the proliferation of T cells, so-called Blastogenic Factors (BF) (22, 23). However, the exact chemical nature of these activities remained obscure. As well, it

was unclear as to whether these activities were primary for the generation of an immune response or were merely amplifying agents that were secondary to the functioning of the system. Moreover, it remained controversial as to whether there were many such factors, or whether one or a few molecules were responsible for all of the activities detected. As well, the cellular sources of these various activities were equally mysterious, in that both T cells (24) and macrophages were implicated (25) (26).

Assays that detected the capacity of T cells to kill target cells through direct cell-cell contact were devised in 1968 by Ted Brunner and Jean-Charles Cerottini and their co-workers (27). Subsequently, Rolf Zinkernagel and Peter Doherty reported in 1974 that cytotoxic T Lymphocytes (CTL) could only recognize specific antigens if expressed by histocompatible target cells (28), a phenomenon that came to be known as MHC restriction of CTL antigen recognition. This finding, and the previous observations regarding the MHC restriction of antibody production, led to an explosive controversy as to the nature of the T cell antigen receptor (TCR), and how it recognized both antigen and MHC-encoded molecules that continued unabated for the next decade.

Then in 1975, Rolf Keissling, Eva Klein and Hans Wigzell, and independently, Ronald Herberman's group discovered a different type of killer cell that did not appear to recognize specific antigens or to be restricted by the MHC. This type of cell became known as the Natural Killer (NK) cell, which was thought to be analogous to the natural antibodies that were found in individuals in the absence of specific immunization. The origin of NK cells remained controversial for more than 2 decades, and only with additional ways to identify B cells and T cells, has it become recognized that NK cells represent a distinct lineage of lymphocytes.

Also in 1975, George Kohler and Cesar Milstein made the surprising discovery that somatic cell hybrids between immunized splenic B cells and murine plasmacytoma cell lines could make continuous quantities of monoclonal antibodies (MoAbs) (29). Thus, Burnet's clonal selection hypothesis was finally proved correct, and his prediction that the proof would rest with the ability to manipulate and study the clonal progeny of a single cell was borne out. The discovery of hybridomas and MoAbs revived the idea of using antibodies as therapeutic "magic bullets". However, the technology to make human monoclonal antibodies proved difficult, and the Human Anti-Mouse Antibody (HAMA) response, which is really a form of serum sickness, effectively precluded the widespread use of mouse MoAbs as therapeutic agents.

In 1976 Doris Morgan and Frank Ruscetti working in Robert Gallo's laboratory reported that culture medium conditioned by proliferating lymphocytes promoted the long-term growth of T cells (30). Subsequently, using conditioned medium containing the T cell growth factor (TCGF) activity, we reported the creation of the first antigen-specific T cell clones in 1979, twenty years after Burnet first forwarded his clonal selection hypothesis (31). Having reduced the tremendous diversity of antigen recognition by the cell population to the progeny of a single cell, for the first time it was possible to prove that antigen recognition by T cells, like B cells, was also clonal. At the time, we predicted that monoclonal T cells would be just as important for future studies directed at determining

the function of T cells as monoclonal antibody producing cells were for understanding the function of B cells. Thus, we felt that ***"the growth of large numbers of monoclonal antigen-specific T cells would lead to the identification and molecular characterization of the TCR, the mechanisms responsible for T cell cytolysis, and the identification of T cell differentiation markers"*** (31)**.**

Monoclonal antibodies reactive with distinct human T cell subsets were generated at the same time that we had generated T cell clones (32), and in a series of reports, Ellis Reinherz, Stuart Schlossman and their co-workers demonstrated that these antibodies were useful in defining helper (CD4+ T cells) and cytotoxic (CD8+ T cells) (33). Subsequently, they discovered that an antibody that recognized a molecule expressed on all peripheral T cells (CD3) blocked T cell proliferation in response to mitogens, while this same MoAb was mitogenic itself (34).

Molecular Immunology

While the 1960s and 1970s were devoted to identifying and isolating the cells responsible for the immune response, the 1980s and 1990s were devoted to discovering and characterizing the molecules involved in promoting the proliferation and differentiation of the various cells, particularly the T cells. With TCGF-dependent T cell clones available, it was possible for us to create a quantitative assay for the TCGF activity (35), which we then used together with standard biochemical methods to identify, characterize and purify the molecule responsible for the activity (36). Subsequently, we generated monoclonal antibodies reactive with the purified molecule, then used these antibodies to purify the molecule to homogeneity for the first time (37). These findings were also the first to show that a lymphokine or cytokine ***activity*** could be ascribed to a single molecule and not several molecules, as had been proposed by others. Moreover, they immediately pointed us to a series of experiments that eventually identified the first cytokine receptor (38) and to the conclusion that cytokines function in exactly the same way as classic hormones, i.e. by binding with high affinity to specific cell surface receptors.

Anticipating the discovery of additional cytokines, in 1979 this new class of molecules were named interleukins, to designate that they functioned to signal ***between leukocytes***. At the time, an activity derived from macrophages had been identified that we had shown functioned to augment TCGF production by T cells (39) (40). Therefore, the macrophage product, which had been termed lymphocyte activating factor, was renamed IL1, and the TCGF molecule was renamed IL2.

The decade of the 1980s resulted in an exponential increase in the amount of information available regarding the molecules of the immune system, and in our understanding of how these molecules function to initiate and regulate the immune reaction, all of which set the stage for immuno-stimulatory therapy. Using antigen-specific T cell clones and hybridomas, and clone specific monoclonal antibodies, the T cell antigen receptor (TCR) was identified and characterized biochemically by Ellis Reinherz's group (41, 42)and by Phillipa Marrack and John Kappler and their students (43). Soon thereafter, Steve Hedrick and Marc Davis and their co-workers isolated the first cDNA encoding one of

the 4 chains of the receptor (44). This information placed the TCR into the immunoglobulin super-family, and revealed that the basic structures of the TCR and immunoglobulins are quite similar. Subsequent studies focused on TCR signaling and gene activation revealed that TCR triggering is obligatory for the transcriptional activation of the IL2 gene (45) (46) (47) as well as the genes encoding the IL2 receptor (IL2R) (48-50). Subsequently, the same TCR triggering was found to be responsible for the expression of additional cytokines as they came to be discovered.

Dendritic cells were discovered, and found to present antigen to T cells with marked efficiency (51). Moreover, B cells were also shown to present antigen effectively to T cells (52). Also, macrophages were found to process protein antigens and present peptides as antigens to the TCR, revealing that T cells recognized fundamentally different antigenic molecules as compared with antibodies (53). Ultimately, the structure of MHC encoded molecules was determined, and for the first time it was realized how antigenic peptides are bound to the MHC molecules and presented to T cells, thereby solving the controversy of MHC restriction of TCR antigen recognition (54). The TCR recognizes a complex of peptide antigen and MHC encoded molecules. This revelation explained for the first time the MHC restriction of immune responses, in that CD4+ helper T cells recognize antigens bound to MHC Class II-encoded molecules, and CD8+ CTL recognize antigen bound to MHC Class I-encoded molecules.

During the 1980s and 90s, almost two dozen new interleukins were discovered, most of them with homologies to IL2. Some of these interleukins, such as IL4 (55), IL6 (56), and IL10 (57), were found to be important for helping the production of antibodies. Therefore, the T cell Replacing factor activity described in the early 1970s was explained in part by the discovery and characterization of these cytokines. Direct cell-cell contact between helper T cells and B cells mediated by T cell derived CD40 ligand and B cell CD40 was also demonstrated to be necessary for optimal T cell help in antibody formation (58). Other cytokines, in particular IFN-γ (59) and IL12 (60), were found to be primarily involved in the generation of classic cell-mediated immune responses, such as the Delayed Type Hypersensitivity (DTH) reaction. IL2 was found to be important for the production and action of both the so-called TH1 cytokines, i.e. DTH cytokines, and the TH2 cytokines, i.e. antibody-related cytokines (61 , 62). IL2 itself became the first interleukin to be discovered at the genetic level by Tadatsuga Taniguchi and colleagues (63), and recombinant DNA technology became the method of choice to identify and characterize the new interleukins.

Two other cytokine families were found to differ structurally and functionally from the interleukins, the interferons (IFN) (64, 65) and the tumor necrosis factor (TNF) family (66). Although closely related to the interleukins, the IFNs are distinct, both structurally and functionally, in that the IFNs have direct anti-viral activity (hence their name as substances that ***interfered*** with viral replication). As the name connotes, TNF is capable of causing the necrosis of tumors (67). However, in addition, subsequent to its identification at the molecular level, TNF was discovered to be one of the primary mediators of inflammation and responsible for much of the toxicity associated with

severe infection, including the systemic inflammatory response syndrome (SIRS), or septic shock (66). Bacterial toxins, such as endotoxin from coliforms and exotoxins from pyogenic organisms were found to be potent stimuli for the production of TNF-α. One of the major functions of TNF-α is to increase vascular permeability, which promotes the extravasation of cells and plasma at the site of inflammation. Thus, the classic signs of inflammation, i.e. rubor, dolor, calor and tumor, in large pert can be ascribed to the effects of TNF-α and the other pro-inflammatory cytokines. When localized, this inflammatory response is beneficial to the host, in that the immune effector cells and serum components can readily access the site. However, if TNF-α is released in large quantities systemically there is a generalized extravasation of cells and plasma, which results in hypotension. Thus, pro-inflammatory cytokines are responsible for the toxicities observed when bacteria and bacterial extracts were administered in attempts to treat malignancies at the turn of the last century. This understanding solved a longstanding controversy as to the origin of the toxicities of bacteria and other microbes. The signs and symptoms of inflammation result from the microbial toxin-induced production of "endogenous pyrogens" (cytokines), and not due to direct effects of the microbes or microbial toxins themselves.

The cellular origins of the various cytokines were identified as these molecules became purified and cloned. Thus, in addition to T cells, NK cells and macrophages came to be known as major sources of cytokines during an immune/inflammatory reaction. Only 3 cytokines were found to be restricted to T cells, IL2, IL4, and IL5. By comparison, IFN-γ is produced by both T cells and NK cells, while TNF-α is produced by T cells, NK cells and macrophages. Macrophages are also the source of other pro-inflammatory cytokines, in particular IL1 and IL6. In retrospect, the original Lymphocyte Activating Factor was most probably a mixture of these macrophage-derived pro-inflammatory cytokines. The cells that participate in the immune/inflammatory reactions are connected with one another via these cytokines, which becomes of major importance when cytokines are administered therapeutically.

The mechanisms involved in the interaction of the cytokines with their respective target cells were found to reside in the expression of high affinity, ligand-specific receptors expressed on the cell surface (38, 68, 69). The IL2R is comprised of 3 chains that cooperate to form a very high affinity receptor (Kd = 10 pM). These high affinity heterotrimeric receptors are expressed transiently only on antigen-selected T cells, thereby conserving the specificity of the immune response. By comparison, NK cells differ from T cells by expressing IL2Rs constitutively. However, unlike T cells, ~ 90% of NK cells lack the α chain of the IL2R (70, 71). This results in IL2Rs comprised of only the β and γ chains, which have a 100-fold lower affinity for IL2 compared with heterotrimeric IL2Rs. This difference between T cell and NK cell IL2R expression eventually became important when IL2 was administered as a therapeutic (72).

Also, in the 1990s, immunoglobulin Fc receptors (FcR) were characterized at the molecular level, and were shown to be the major molecular connections between the humoral immune response and the cellular immune system of phagocytes, as proposed originally by Metchnikoff (73). In addition, B cells and NK cells were found to express

FcRs, and the function of these special FcRs are now know to be important in the regulation of the production of antibodies, as well as the function of cytolytic cells, via a phenomenon termed Antibody-Dependent Cellular Cytotoxicity (ADCC).

History of the Use of IL2 in the Clinic: High Dose, Intermittent Therapy for Cancer.

In the early 1980s before IL2 was available in pure form and in large enough quantities for therapeutic use, Steven Rosenberg attempted to use lymphocyte conditioned medium containing TCGF (IL2) activity to generate large quantities of tumor-specific lymphocytes *in vitro*. He intended to then re-infuse these cells, as adoptive immunotherapy for the treatment of cancers that were refractory to conventional radiotherapy and chemotherapy. Unfortunately, it was difficult to generate tumor-specific CTL reactive to autologous tumor cells, so that Rosenberg resorted to simply culturing PBMCs from his patients for several days in lymphocyte conditioned medium, then re-infusing the *in vitro* cultured cells that he termed Lymphokine-Activated Killer (LAK) cells. Concomitantly, the lymphocyte-conditioned medium was also administered. The identity of the LAK cells, whether T cell, NK cell, or monocyte, remained obscure.

As purified natural IL2 became available, soon followed by purified recombinant IL2, Rosenberg together with Michael Lotze and others from their group began using IL2 as a continuous infusion together with the cultivated LAK cells as immunotherapy (74). Based upon experimental tumor models in mice, they used the principles of cytotoxic chemotherapy of dose intensification to toxicity to establish their IL2 dosing regimens (75). Thus, doses of 150 million Units of IL2/day, or ~ 10 mg of recombinant IL2 protein, became standard. This total dose is administered as an intravenous bolus in three divided doses daily every 8 hours for 3-5 days, and still is in use today, having been approved by the FDA for the treatment of renal cell carcinoma and malignant melanoma.

The initial 25 patients treated with high doses of IL2 together with LAK cells were reported to undergo either a partial or complete remission rate of ~ 40%, an unprecedented response rate (74). Subsequent studies by many investigators revealed that the LAK cells added no benefit to the IL2 therapy and the LAK infusions were abandoned. Now, 15 years later, several hundred patients have received this treatment, and the overall results reveal that 9% of patients enjoy a long-term (i.e. > 5 years) disease-free remission, while 10% achieved a partial remission, with > 50% decrease in measurable tumor mass (76).

The mechanisms responsible for the anti-tumor effects of high dose IL2 therapy remain obscure to this day. Rosenberg has maintained that the severe, clinical grade III-IV toxicity is necessary for the anti-tumor response (76). However, 100% of the patients experience the toxicity, while only 19 % achieve an objective diminution of tumor mass. The toxicities experienced are remarkably similar to those symptoms described for the SIRS, or septic shock. Thus, patients experience high fevers, 104-5°F, with severe rigors, followed by hypotension and hypoxemia. Accordingly, this therapy is administered in the hospital, usually in an intensive care unit, and most patients require assisted ventilation and blood pressure support in the form of IV fluids and vasopressors. Most patients who undergo high dose IL2 therapy gain ~ 20 Kg of body weight in the form of fluid.

BIOLOGICAL EFFECTS OF IL2

IL2 is the principle growth factor for T cells *in vivo*, and as well serves as a growth factor for NK cells (77, 78). In addition, IL2 promotes the differentiation of both T cells (both TH and CTL) and NK cells, serving to promote the differentiated function of these cell types. Accordingly, IL2 is responsible for the expansion of the absolute number of mature antigen-selected T cells, and the number of NK cells. Moreover, the differentiated functions of these cells, i.e. the secretion of cytokines and the capacity to effect cytolysis, are promoted by IL2. Because these effects of IL2 set in play a cascade of additional cytokines and cytolytic effector molecules, IL2 has the potential of eventually leading to a severe inflammatory reaction. In essence, the IL2 produced by antigen-stimulated T cells acts to expand the number and function of these cells, but as well, by activating NK cells, IL2 communicates between the antigen-specific, acquired immune response, and the nonspecific innate host defenses mediated by NK cells and macrophages. Upon IL2 activation, NK cells produce a restricted subset of cytokines that include Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), IFN-γ, and TNF-α. These cytokines, in turn, are potent stimuli for monocytes and macrophages. Accordingly, the stimulation of macrophages that occurs indirectly via the IL2-activation of NK cells eventually results in the production of cytokines by macrophages, in particular the pro-inflammatory cytokines TNF-α, IL1 and IL6 (79). Moreover, the macrophage production of IL12 then feeds back to further enhance the function of the NK cells, thereby creating a circuit that can promote itself to create the inflammatory response. The NK cell-derived monocytotropic cytokines also enhance macrophage antigen processing and presentation via MHC molecules, as well as antigen clearance via potentiation of Fc receptor expression.

IL2 also imparts survival signals to its target cells, mediated via the activation of the expression of a set of genes that encode molecules, such as Bcl2 and related family members that are anti-apoptotic (80). However, if IL2 is withdrawn from IL2R+ cells, the expression of these genes is extinguished, and subsequently the cells undergo programmed cell death. This "cytokine withdrawal apoptosis" is a major phenomenon regulating the size of the expanded pool of antigen-selected cells, particularly after antigen is cleared and no longer promotes the production of IL2 (81).

When antigen persists, even though the immune system has been activated, a phenomenon termed Activation-Induced Cell Death (AICD) ensues, which also leads to apoptosis (82). It has been shown that this form of programmed cell death is mediated via the activation of the FasL/Fas pathway (83)as well as the TNF-α/TNF-αR pathway (84), and is dependent upon both TCR triggering and IL2R activation. At present the physiological significance of this phenomenon remains obscure, but some have proposed that it underlies the mechanisms responsible for peripheral tolerance, as well as to the phenomenon of "high zone tolerance"(85).

IL2 PHARMACODYNAMICS AND THE THERAPEUTIC INDEX

At the time that Rosenberg and Lotze first used high-dose IL2 therapy in the mid 1980s, it was not immediately obvious as to why there should be so much toxicity generated. Theoretically, low IL2 concentrations should fully saturate the high affinity IL2Rs expressed by antigen-activated T cells, and higher doses should have no further effect. However, it was not appreciated that most NK cells expressed a different type of IL2R compared with antigen-activated T cells. Also, it was not appreciated that there are ~ 1 billion (10^9) circulating NK cells. Understanding the effects of IL2 therapy has required a detailed determination of IL2 pharmacodynamics, as well as pharmacokinetics. Moreover, the determination of the interaction between IL2 target cells and other cells responsible for the generation of the inflammatory response was necessary to fully comprehend why high doses of IL2 are toxic, and therefore, as to how to effectively use IL2 in the clinic.

IL2 only directly effects target cells that express IL2Rs (i.e. T cells and NK cells). However, because these target cells produce bioactive cytokines that can have wide ranging effects on many tissues, it is important to understand the pharmacodynamics of IL2, which are dependent upon the type and distribution of the IL2Rs. As described, the only cells that express IL2Rs are antigen-activated T cells, and NK cells. The T cells express high affinity (Kd = 10 pM) heterotrimeric IL2Rs, which bind and respond to IL2 in the 1-100 pM concentration range (15 pg/ml-1.5 ng/ml)(68, 69). It is convenient to express the plasma IL2 concentrations in Molar concentrations because the equilibrium dissociation constants of the receptors are expressed in these units of measure. However, as doses of drugs are usually expressed in protein weights, or units of biological activity, it can be confusing when trying to understand the pharmacodynamics and pharmacokinetics. In this regard, it is helpful to make the calculation of Molar concentration into molecules/ml, using Avagadro's Number (6.02×10^{23} molecules/mole). Thus, 1 pM = 10^{-12} moles/L = 600 million molecules/ml. Therefore the effective range of concentration of IL2 for T cells in molecules/ml is 600 million molecules/ml to 60 billion molecules/ml.

By comparison, the equilibrium dissociation constant of the intermediate affinity IL2R expressed by ~ 90% of NK cells is 100-fold lower than the high affinity IL2R, i.e. Kd = 1 nM. Accordingly, the IL2 concentration range that binds to this class of IL2Rs varies from 100 pM to 10 nM, which in molecular terms is 60 billion molecules/ml to 6 trillion molecules/ml. The difference between the high affinity and intermediate affinity IL2R resides in the absence of the α chain (86). Thus, the intermediate affinity IL2R is comprised of only the β and γ chains, and thus these receptors lack the fast association rate imparted by the α chain.

The therapeutic index is influenced by these two distinct receptor classes, in that 100-fold higher doses of IL2 are required to generate IL2 concentrations high enough to bind to the intermediate affinity IL2Rs expressed by the majority of NK cells. Since there are ~ 10^9 circulating NK cells, most of the systemic toxicity can be avoided if the doses of IL2 are kept low, resulting in IL2 concentrations < 100 pM. This results in the activation of <

10% of the intermediate affinity IL2Rs expressed by NK cells, while saturating the high affinity IL2Rs expressed by antigen-activated T cells (87, 88). Whether or not systemic toxicity is produced at any IL2 concentration is dependent upon how many cells express IL2Rs, in that the absolute number of cells that produce secondary proinflammatory cytokines, such as TNF-α, IFN-γ, and GM-CSF essentially determines how great the symptoms of the SIRS will occur. Accordingly, if there is a systemic persistent infection as occurs in untreated HIV infection, one would expect greater numbers of antigen-activated T cells to be present. Therefore, lower doses of IL2 may produce toxicity in this setting than in a situation when the number of IL2R+ cells is lower.

IL2 PHARMACOKINETICS

IL2 is a small (15 kDa) globular glycoprotein. Therefore, when injected IV, IL2 rapidly passes between capillary endothelial cells and distributes into total extracellular space, which in a normal adult is ~14 L (89). When injected either subcutaneously or intramuscularly IL2 is rapidly taken up by the lymphatics, and distributed via the circulatory system to total extracellular space. Subsequently, IL2 is filtered via the glomeruli, then reabsorbed by the tubular epithelial cells and metabolized. Consequently, it is cleared fairly rapidly.

The half time for distribution after an IV injection is ~10 minutes. Therefore, within 40 minutes >94% of the injected IL2 is distributed into the 14 L of extracellular space. This explains why in the initial experiments from Rosenberg's group, the IV bolus doses of IL2 disappeared from the circulation so rapidly, and why they attempted to counteract this phenomenon by injecting higher and higher doses. Once distributed into the extracellular space, the renal clearance of IL2 proceeds with a t ½ = 2 ½ hours. Therefore, > 94% of the IV administered IL2 is cleared within 10-11 hours.

After a subcutaneous injection, IL2 is absorbed into the lymphatics and appears in the blood with a t ½= ~ 50 minutes, and peak plasma IL2 concentrations are attained by 2-3 hours (89). Thereafter, the same rate of renal clearance ensues as found after IV administration, and by 12-14 hours only very low levels are detectable. Accordingly, to achieve a constant IL2 concentration within the extra-cellular space, the administration of IL2 every 12 hours would be ideal. In practice, we find that daily subcutaneous administration of 1.2 mU/m^2 BSA is sufficient to achieve an ongoing IL2 response. This dose of IL2 results in a peak plasma IL2 concentration of 20-30 pM, which is high enough to saturate ~ 70% of the high affinity IL2Rs on antigen-activated T cells, but will only bind to ~ 2-3% of the intermediate affinity IL2Rs expressed by the majority of NK cells.

IL2 TREATMENT REGIMENS: INTERMITTENT vs. CONTINUOUS THERAPY

At present, there are two schools of practice regarding dosing regimens. As the initial dosing as used by the oncologists employed intermittent ultra-high doses of IL2, this regimen has been maintained and has been approved by the FDA for use in the treatment of renal cell carcinoma and malignant melanoma. The daily dose amounts to 150 million

U (10 mg), given in 3 divided bolus IV injections. This regimen is administered for 3-5 days, and repeated within 6-8 weeks if there is an objective response but not a complete disappearance of detectable tumor.

For the treatment of HIV infection, Cliff Lane and colleagues have also used intermittent administration, but the dose has been reduced ~ 10-fold from that used in cancer therapy, to 15 million U/day (1 mg/d) (90, 91). This dose is divided into twice daily dosing and is either given as a subcutaneous injection or as a continuous IV infusion. An intermediate dose of 9-10 million U/day (~ 600 µg) has recently been described for the treatment of HIV infection, given in two daily doses subcutaneosly.

When these high intermittent dosing schedules are used, during the treatment interval a marked lymphopenia is observed, and all circulating mononuclear cells decrease to very low levels. Simultaneously, a capillary leak syndrome occurs, and hypotension can be a problem during this interval. After the cessation of IL2 injections, a rebound lymphocytosis occurs, with all circulating mononuclear cells increasing to concentrations 10X the pre-treatment values. Subsequently, over the ensuing weeks the lymphocyte and monocyte concentrations gradually return towards the pre-treatment values. Thus, high dose intermittent IL2 administration results marked cellular and fluid shifts, and it is difficult to determine whether there is actually a net gain or loss of lymphocytes with this regimen.

The low dose continuous dosing regimen was developed by us in an attempt to arrive at a dose and schedule that was non-toxic, and also would provide for fairly continuous exposure of the cells to IL2 (88, 92). Continuous IL2 exposure, rather than intermittent dosing was considered preferable to maximize the IL2 cellular signaling. The rationale is based on a series of experiments where we found that the T cell proliferative response was proportional to the IL2 concentration, the IL2R density, and the duration of the IL2/IL2R interaction (45, 48). Accordingly, if antigen-activated T cells expressing optimal levels of IL2Rs are exposed to an IL2R saturating concentration, i.e. ~100 pM, the magnitude of the proliferative response is directly dependent on the duration of the IL2 exposure. In addition, the withdrawal of IL2 from IL2R+ cells results in rapid apoptosis, such that the cells are irreversibly damaged within 12-24 hours without IL2. Therefore, exposure to IL2 intermittently may well create a situation where recently antigen-activated IL2R+ cells are actually killed by withdrawal of the survival-enhancing properties of IL2.

THERAPEUTIC USES FOR IL2

Intermittent IL2 therapy at all of the high doses that is used primarily to promote rapid changes within the immune system, either for the treatment of cancer, or in situations where there is a need to enhance the number of circulating lymphocytes rapidly. As already mentioned, the ultra high IL2 dose used in cancer treatment does result in long-term disease-free intervals for a small number of patients. In patients infected with HIV, the high dose and intermediate intermittent IL2 doses can rapidly increase the concentration of circulating CD4+ T cells, which then can remain elevated for several

months after the treatment interval. However, whether these changes in the concentrations of circulating CD4+ T cells translate into a clinical benefit remains unknown. Consequently, currently, there are two large-scale clinical trials on-going that are designed to determine the answer to this question.

The problem with all of the higher IL2 doses that are used intermittently is one of toxicity. The systemic side effects generated, including fatigue, fever, rigor, and capillary leak, are remarkably similar to the symptoms and signs described a century ago, when bacteria and bacterial extracts were used in cancer treatment. Because of these toxicities, a wider range of illnesses where it might be beneficial to augment the function of the immune system are precluded. It is important to realize that the toxic side effects are IL2 dose-dependent, which accounts for the progressive decreases in dosing that has occurred since the initiation of the ultra high dose used for cancer treatment.

Low doses of IL2 can be given daily for prolonged intervals. We have administered low dose IL2 to asymptomatic HIV+ individuals for as long as 5 years without significant adverse events (89). In particular, we have not experienced the development of autoimmune phenomena, a danger that many have thought probable with continuous IL2 therapy. We now have experience with ~ 100 individuals treated continuously with low dose IL2 for at least 1 year, and we find that the earliest change detectable in the immune system is a 5-10-fold increase in eosinophils within the first two weeks of initiation of therapy. Thereafter, the next change that is readily detectable is a progressive increase in the concentration of circulating NK cells. The NK cell concentration increases by a mean of ~ 200 cells/µl over 2 months, then stabilizes at this increment as long as IL2 is administered. In asymptomatic HIV+ individuals treated with Highly Active Anti-Retroviral Therapy (HAART) and low dose IL2 for 1 year, there is an increase in the concentration of circulating CD4+ T cells at a rate of ~ 10 cells/month. This rate is ~ 2.5 times faster than reported for HAART alone. A randomized, controlled trial testing the effectiveness of IL2 for the acceleration of the recovery of the immune system is presently in progress. The preliminary data analyzed after 6 months of combined HAART + IL2 has revealed a significant difference in the rate of increase in the concentration of naive CD4+ T cells and in the concentration of NK cells. Whether these changes in the concentration of circulating lymphocytes will translate into a clinical benefit remains to be determined.

From our earliest experiments it was clear that IL2 is effective in promoting T cell proliferation only after antigen activation of IL2R expression (77). Moreover, IL2R expression was transient. Upon removal of the antigen, a progressive decline in the density of IL2Rs is detectable, such that after 14 days in culture, most cells lose expression of the IL2R and become unresponsive to IL2 (48). However, if antigen is re-introduced, the cells rapidly re-express IL2Rs and once again become IL2-responsive. Accordingly, the proliferative response to IL2 is antigen-dependent, and antigen non-reactive cells will not expand, even though IL2R saturating concentrations of IL2 are present. Extrapolating these *in vitro* findings to clinical situations, it follows that it will be beneficial to introduce the desired antigen at the time that IL2 is administered to expand the number of antigen-specific cells.

Recently, we have used the interruption of HAART in HIV+ individuals, combined with low dose daily IL2 therapy (93). We have found that plasma virus becomes detectable within 19 ± 3 days upon cessation of HAART. Thereafter, there is a rapid increase in viral concentration (doubling time 1.6 ± 0.1 days), to a peak concentration after a mean of 17 days. Subsequently, there is a progressive decline of detectable plasma virus with a t ½= 3.5 ± 0.7 days, with a trough concentration that is ~ 10-fold lower than the peak reached in 18 ± 3 days. Coincident with the viral relapse, there is a CD8+ lymphocytosis, which peaks at ~ 2-fold the baseline concentration just after the peak of viremia. Subsequently, the CD8+ T cell concentration remains elevated as the virus progressively decreases. Moreover, the rate and magnitude of viral decline correlates with the magnitude of the CD8+ lymphocytosis.

These data are consistent with the interpretation that the viral relapse activates the CD8+ memory T cells to become IL2R+, and the IL2 administered then promotes their expansion. However, we need to do additional experiments to support this hypothesis. In particular, experiments are indicated to identify and quantify the number of HIV-specific T cells, and as well, to evaluate whether the increase in the concentration of CD8+ T cells is due to their proliferative expansion. However, these data support the notion that antigen + IL2 will be more effective in stimulating an antigen-specific immune response than either antigen or IL2 alone, and that the next step will be to combine IL2 therapy with a therapeutic immunization schedule.

The principles that we have used to develop low dose, non-toxic daily IL2 administration now permit the extension of this therapeutic dosing and regimen to other infectious diseases, and as well, to cancer. Accordingly, we are developing protocols to test the efficacy of the addition of low dose daily IL2 therapy to the standard therapy for the treatment of Hepatitis C Virus (HCV) infection, which consists of IFN-α and Ribavirin. In addition, these same principles can now be applied to the use of IL2 as an adjuvant given in combination with vaccines. Most adjuvants in use today are focused on enhancing antigen processing and presentation by antigen-presenting cells. However, if IL2 production is limited or compromised in any way, the resultant proliferative expansion of antigen-selected cells will necessarily be abbreviated. Accordingly, IL2 adjuvant use may well markedly improve the immunogenicity of weak vaccines.

COMBINATION ANTIBODY-IL2 THERAPY

As discussed in the introduction, the use of mouse MoAbs as therapeutics has been hampered by the human anti-mouse antibody response (HAMA). However, over the past 20 years genetic engineering approaches have succeeded in "humanizing" the mouse MoAbs (94). Thus, the mouse variable regions are retained, while the rest of the antibody, i.e. the constant regions are replaced with human sequences. Even, better, the antigen-binding Complementarity Determining Region 3 (CDR3) of the mouse antibody is the only part that is not of human sequences. These approaches have demonstrated the "proof of principle" that it is possible to markedly diminish the HAMA, so that MoAbs are once again feasible to consider as therapeutics.

More recently, transgenic mice have been created that express only human genetic sequences (95-97). These mice are remarkable, because they contain almost the entire genomic DNA encoding both Heavy and Light chains, but they are tolerant to the human gene products because they have been exposed to the human antibodies during embryogenesis. Thus, their entire antibody production is from human sequences, yet the can recognize all human molecules as foreign. Consequently, it is now possible to create human MoAbs reactive with all foreign molecules, and as well, all human molecules. Accordingly, the prospects future therapeutics using human MoAbs now appears limitless.

Anticipating the future, the combination of human MoAbs with cytokines like IL2 should provide for an enhanced efficacy of the MoAbs. IL2 will function to augment the capacity of the reticuloendothelial system to recognize antigen-antibody complexes by its effects on NK cells and macrophages. Thus, by stimulating NK cells to release IFN-γ, GM-CSF, and TNF-α, these cytokines will increase the cell surface density of Fc receptors, as well as the phagocytic capacities of the cells. Therefore, the effector arm of both the humoral and cellular arms can both be artificially enhanced for the first time. The net effect will be to improve the efficiency of MoAb therapy, so that a maximal response may be obtained with lower doses of MoAbs. As well, the frequency of MoAb administration may be influenced by the use of immunomodulating cytokines like IL2.

CONCLUSIONS

At this juncture, we stand on a new therapeutic threshold. The separation of the immune response into a humoral and cellular school resulted in a century of reductionist science that has finally given us a fairly complete picture of the cells involved in the system, and of the molecules that direct their activities. Armed with cytokines, the hormones of the immune system, and the knowledge that they mediate their effects via interacting with high affinity stereospecific cellular receptors, it is now possible for the first time to design rational therapeutic strategies designed to improve the function of the system without generating undue toxicities. Moreover, the principles of therapeutic cytokine administration can now be applied together with vaccines to maximize immune responses after immunization. In addition, with the availability of human MoAbs, for the first time it will be possible to harness the power of the specificity and diversity of the immune response, to direct therapy at specific molecules and cells. These advances should transform medicine, as we presently know it.

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