Interferon.

Interferons are a group of similar proteins, part of a larger family of immune response mediators now known as cytokines. Interferon is induced in viral infected immune cells and in the human and animal body as the first line of defense against virus infection. Different classes of interferons are produced by unique cells of the immune system. Interferon itself does not act directly on the virus, but activates a number of different biochemical pathways, all directed towards inhibiting virus production. That is, if one added interferon, directly to a test tube with virus in the absence of cells it would have no effect on subsequent virus replication assuming all the interferon had been removed. Scientists originally hoped that interferons could be used clinically as a general anti-viral and anti-cancer agent like antibiotics, but its use has been limited to treatment of a few types of cancers, and to a few viral diseases such as hepatitis C Newer anti-viral drugs are more effective and with less side effects.

I became aware of interferon while a post-doctoral fellow in John Holland's lab at University of California, Irvine. John had published a paper on interferon in the 1960's, probably as part of his Ph.D. or post-doctoral work. When I moved to Indiana University in 1967 I had not planned to work on interferon in my lab, because I felt this area of research was already too crowded, and I was not convinced at that time of interferon's importance. When Barbara Cordell a graduate student, discovered that the addition of viral double stranded RNA, an intermediate of virus replication, to cells resulted in cell death, a hypothesis was that interferon was involved. Our tests for its production were negative. In retrospect this was surprising, since double stranded RNA definitely induces interferon. However the cells we were using were not immune cells.

An early post-doctoral student in my lab was Bob Fleishmann. Bob had graduated with a Ph.D. from Purdue University in 1972 from the laboratory of Dr. Ed Simon, working on the induction of interferon by Newcastle Disease Virus. Bob was a well-built, red headed young man, and arrived with his wife, Christina (Chris) who found a position in the department as a lab technician. They were a very devoted couple. She later obtained a Ph.D. at the age of 40 but did not live very long to enjoy her success. She died a few years later of breast cancer.

Bob joined my lab to work on purine metabolism an ongoing project, but he could not shake of his addiction to interferon research. I was influenced by the constant talk and discussion with Bob about interferon and its potential. However, during his stay in the lab, I avoided working with interferon. Bob was a post-doc for only a short time and left prematurely to establish his own lab in Galveston, Texas. He eventually became an editor of the Journal of Interferon Research and very influential in this area of research.

During these early years at IU my research area was predominantly on the isolation of mammalian cell mutants defective in urine metabolism, and the development of chromatographic methods for the separation and quantitation of nucleotides, components of DNA and RNA. We examined whether Bloom's syndrome resulted from nucleotide imbalance. Individuals with this inherited condition are small in stature, are sun sensitive, and have a high frequency of cancer. We hypothesized that this may be due to a lack of specific nucleotides, or a defect in the purine biosynthetic pathway. This hypothesis was wrong, Bloom's syndrome was later found to result from a defect in an enzyme (helicase), involved in DNA structure and DNA stability.

Mexico City and High Pressure Chromatography

In the early 1980's Hector Martinez Valdez, a graduate student from Mexico joined the lab. We met at a scientific meeting in Mexico City and I invited him to join my lab for a short period to study High Pressure Liquid Chromatography and develop techniques for the purification of nucleotides in collaboration with an expert on chromatography, Dr. Raman Kothari from India, who had previously worked for two years in my lab. Hector remained 2.5 months in the lab resulting in two publications on HPLC methods, and then returned to Mexico to complete his Ph.D. and MD degrees.

Shortly after I was invited, by Hector to teach a course on HPLC techniques at the National University in Mexico City. On the day of my arrival the Mexican Peso collapsed, going from 8 to 80 to the dollar. This was during one of Mexico's recurrent economic crisis. I stayed with Hector and Patty in their small apartment since the university department discovered after inviting me that it could not afford a hotel or even pay for my services due to the devaluation. This did not distract from my enjoying the city and its great restaurants, including a famous Polish restaurant visited a few days earlier by the then reigning Pope. Hector and his wife Patty were extremely hospitable, and we fast became close friends.

The course itself was not a great success because of the lack of specific equipment. I had asked whether I should bring spare parts with me, and insisted that the organizers test that everything worked, but on arrival I discovered that essential components of machinery were lacking. The course was scheduled to begin at 9.0 a.m., the students would arrive for my lecture around 10.00. I felt that the whole course was a fiasco. This was Mexico! I recall that I gave a few lectures, but there was no hands-on work as promised. No one seemed to care.

I enjoyed the stay in Mexico City, and was joined by my son, Yuval and later the rest of the family, thus making a vacation out of it. I remember this visit quite vividly since Yuval arrived in an old grungy overcoat much too large for him. He was a student at Princeton and apparently this was the fashion among the undergraduates. His coat was I suspect third or fourth hand and looked the worse for wear. He looked like a bedraggled refugee alighting from the plane, and I felt like sinking into the ground since the outside temperature must have been in the 80's. How could I introduce my son to this well-heeled group of Mexicans! We all disliked the overcoat and luckily it was too hot to wear. A few months later Yuval spent the summer vacation in Bloomington and Mimi would not allow the coat into the house, thus it stayed in the garage. At the end of the summer Yuval returned to Princeton minus the coat, and Mimi took the opportunity to place it in the garbage. He has never asked to this day what happened to his coat. Just as well it was forgotten.

I actually have great memories of that trip and stay in Mexico City, and the visit to the Volcanoes and surrounding area. Hector was a great guide.

Heat shock proteins and interferon

On returning to the lab we embarked on a new project. This was a period of great interest in many labs on "heat–shock" proteins. These were proteins that were induced very quickly (10-30 minutes) when mammalian cells were exposed to a few degrees above normal temperatures. In a water bath They could be identified as discrete bands on poly-acrylamide gels by electrophoresis. These proteins were normal components of the cell, but their synthesis was induced on exposure to temperatures above 37C.. Their function was unknown and hypothesized to be involved in cancer and/or the immune response. These proteins were also induced by chemicals that caused cellular stress. We thus asked

the question whether interferon was basically a heat shock protein, since it was known that stress responses led to the interferon production. We now know that all organisms including bacteria contain genes that code for heat shock proteins, and these are involved in maintaining the integrity of other proteins. Some of the heat shock proteins are over expressed in cancer and are thus a signal of aberrant growth. These proteins have been given the name chaperones, since they interact with other proteins to maintain stability and shape.

Hector returned to my lab after completing his graduate studies wanting to work on an immunology related project, and we thus decided to look at Bloom's syndrome lymphocytes, normal lymphocytes and a variety of cell lines to determine whether small increases in temperature of incubation induced interferon

We initially found that two cell lines, a normal Epstein Barr (EB) B virus transformed B-cell line, and a Bloom's syndrome B-cell line (also transformed by EB virus) were incubated at 40^{o} C for up to 4 hours, that an antiviral activity could be detected in the media. This antiviral activity appeared to be due to the production of very low levels of interferon–gamma that synergized with spontaneously produced alpha interferon or some other antiviral material of unknown property. We performed a series of experiments to characterize the products. The results indicated that the lymphoblastic cell line produced IFN-alpha spontaneously and IFN-gamma following heat treatment. The antiviral activity was reduced by both anti-IFN-alpha and anti-IFN-gamma antibodies confirming that both types of interferon were present. The experimental data appeared to be very clean and unambiguous. We now know that there are many more types of interferon than we knew of then, and some of the anti-viral activity might have been due to unknown interferons. These however would not have been inhibited by the anti-serum antibodies we were using.

There was no heat induction of interferon or anti-viral activity in any other cell lines that were tested (those were all either epithelial or fibroblast cell lines) suggesting that this phenomenon was unique to Epstein Barr transformed B-cells. These results were published in the influential Proc. Natl. Acad. Sci. USA. Although the work seemed very thorough and convincing, on rereading the paper I have the impression we ourselves were not a hundred percent convinced that the inhibition of virus was due to interferon gamma. Also, the amount of anti-viral activity induced was very low, compared to what is normally found in human cells after infection by virus.

At this time, I left the lab in the capable hands of Hector and embarked on a Sabbatical to Israel and then later Italy. The Weizmann Institute had a large group working on interferon, headed by renowned scientists such as Michel Ravel, Menachem Rubinstein and others. Although I was not working with them I brought cells, and the "heat shocked" material with me. I wanted to confirm that our results were really true. One of the principles of experimental science is confirmation of the results by other groups. I also thought there would be interest at the Weizmann in our data. However, all attempts to repeat the experiments at the Weizmann failed. I cannot remember whether there was some background interferon-alpha, but we could not detect anything after heat shocking the cells. Likewise, the material I brought with me did not contain interferon, or if it did very low levels. I had others do experiment for me, and again there was no induction. I wrote to Hector, that someone else other than T should do the experiments. I was completely puzzled by the lack of reproducibility. When I arrived in Italy I again performed the same experiments without success. At one time Mimi had to return to the US and brought back some cells. Again, I could not persuade them to respond to the heat treatment.

On my return from Sabbatical we attempted to repeat the experiments. They always seemed to work for T, that is, he always induced anti-viral activity but not when I or Hector or a new visiting scientist, Louisa, who had joined the lab from the Weizmann, followed T's protocol. I must admit that I was afraid to go to my office in the morning and face the wrath of Hector and Louis about something T had or not had done correctly. He was always the butt of their complaints. I felt that the morale in the laboratory was becoming very bad, and I had no alternative other than to fire T. I could not believe that anyone would deliberately make up data to curry favor with others or myself. That did seem the situation (I did see the same phenomenon later on with another student). This was all very embarrassing. I requested that our paper be withdrawn and was told that there was no mechanism for withdrawing a published paper at PNAS. I reported my "revised" data to the Journal of Interferon Research in a short communication but this was not accepted, and I was referred back to the original journal. To this day I do not know what really happened. I was embarrassed in front of my interferon colleagues, and for a time found it difficult to live it down. I did not cheat, nor knowingly publish fake data. I find it difficult to believe that anyone would. The observation of heat induction of interferon in hindsight was not all that important, and some in vivo work being done in parallel in the lab by another graduate student supported the in vitro work. However, we could conclude that interferon alpha and gamma are not heat shock proteins. Others have reported similar observations in cells derived from mice and suggest that heat may enhance the activity of interferon, rather than its production, something we did not consider. More recent papers have indicated that this may have been a real phenomenon, but an unusual type of interferon.

Jim Downing, a combined MD-Ph.D. student, decided with my blessing to examine whether there was any relationship between fever, body temperature and interferon induction. This work was performed in collaboration with Professor Elizondo of the Department of Physiology who had the required animals and equipment. Initial experiments were performed on three moneys housed in the physiology department. They were restrained on chairs, and their ambient (surrounding) temperatures increased to 45 degrees so that body temperature rose by 2 degrees. The results were confusing in that an anti-viral material was induced into the plasma, but by our criteria, testing with specific anti-bodies it was not a known interferon. However, blood cells collected from the animals " heat shocked" produced 10-fold more interferon on induction with a plant substance known to stimulate immune cells or following treatment with a bacterial extract also known to induce interferon than in control animals. This anti-viral substance appeared to be gamma interferon. Similar results were achieved when fever was induced by non-viable bacterium. Thus, in rhesus monkeys there is a link between fever and interferon gamma priming although not direct induction. It is possible however that other types of interferon, unknown at the time were being induced.

Jim performed the most audacious experiments on human subjects (of course with permission from the human subjects committee). He convinced fellow graduates to sit in a hot tub (in the physiology department) at a water temperature of 40-45 degrees C, with an inserted rectal thermometer connected to a device that recorded body temperature every 30 minutes. He collected blood samples for immune cell isolation and performed interferon assays at intervals as body temperature rose. The threshold for maximum changes in the white blood cell population appeared at 39^{o}C. The levels of IFN-gamma in the blood did not change although the white blood cells appeared to be primed to make interferon if

challenged. From these studies we concluded that there was a relationship between fever and interferon, but the relationship was not a direct one. Further experiments were performed with Rhesus Monkeys and similar results were obtained when fever was induced by natural or hormonal means. At that time, we were only aware of IFN alpha, beta and gamma. Since then many other classes of interferons have been discovered as well as many other cytokines, involved in the immune response, some of which might have had anti-viral activity. Our inability to understand the data resulted from our ignorance of the mechanism of the immune response and the complexity of the interferon response.

I was now ready to abandon the interferon field, but that was not to be.

While on Sabbatical in Italy, although working in Raul Perez Bercoff laboratory on picornaviruses, I found that nearby (physically) was an interferon laboratory headed by Dr. Fernando Dianzani, highly respected in the interferon/cytokine field. I spent my last few days in Rome talking to him, and I constantly met him later at interferon meetings. I also interacted with a group at the Sanita, (the Italian equivalent of the NIH) working with interferon and cancer headed by Giovanni Rossi, with whom I became good friends. Unfortunately, Giovanni died a few years later of lymphoma. He kept it hidden from his colleagues and students until near the end of his life. I would later spend another sabbatical, seven years later, with one of his students, Philipo Bellardelli, who inherited his mantle. Moreover, a post-doctoral student from the University of Messina also joined my lab, and she came from an interferon background, thus circumstances kept me active in this area of research.

On my return from Sabbatical in Italy/Israel I had a series of individuals join my research group for short times. The first of these visitors was Louisa Chen from the Weizmann Institute. She actually was a technician in Michel Ravels' lab

and had developed techniques for the assay of interferon. She was a very enthusiastic lively middle- aged woman. She and Hector tried to solve the problems of the irreproducibility of heat shock interferon but were unable to do so. Louisa only stayed a year and was a useful resource person. She was followed by Daniela from Sicily. Since her background was virology she wanted to work in the area of herpes virus biology. We examined whether white blood cells from patients with genital herpes were more prone to produce interferon than uninfected controls. We were unable to find any differences, thus bringing this project to a halt. Daniela returned to Sicily and I have had no contact with her since.

Raneiro De Stasio and Interferon-herpes research

A year following my return from Rome, Raniero De Stasio, a student from the department of virology in Rome joined the lab. I met Raniero while working in Raul's lab. I knew him well, and we became friends of his family: mother, father, brother and sister. His mother made Mimi and I costumes for Carnival while in Rome, I an American Indian and Mimi a "Dutch girl". have written about Carnival in Rome elsewhere.

We were also invited to the De Stasio country house (ancestral home) near Caserta. This was a small house, very primitive, in the hills North of Naples. Despite not having much of a kitchen Mrs. De Stasio cooked an excellent lunch and made us feel like family. I don't think she was very happy with the idea of Raniero following me to the USA which had already been discussed. When I left Rome, I handed over my old green fiat (described elsewhere as a "Red Brigade" getaway car) to Raniero's brother, who immediately "totaled" it. Raniero's father, Dr. De Stasio was a microbiologist at the University of Rome. I did not know him well, since he and his wife had temporarily separated during the time we were there. I believe they later reconciled (but eventually separated later). Raniero's

sister “Pupa” left Italy in 1999 and is a professor of physics at U. of Wisconsin, Madison, and for a time was head of their cyclotron.

Raniero wanted to pursue his PhD on a virology project, and since Daniela was working with herpes virus (indirectly) we decided to look at the effect of interferon-gamma on Herpes simplex (HSV) type 1 replication. This was a complicated project since HSV has a complex replication cycle. Its replication is divided into early, middle, and late gene expression, each part of the cycle controlled by genes expressed in the previous part of the cycle. Raniero’s work indicated that interferon-gamma inhibited HSV replication at a very early step of replication after the viral DNA had penetrated the cell. However, we were unable to pin point exactly where in the complex this occurred. Later reports showed that HSV in turn could inhibit the activity of IFN-alpha but apparently not IFN-gamma. As far as I am aware the mechanism of inhibition of HSV by IFN-gamma has never been solved.

Jump ahead a number of years. Raniero married in Bloomington, an Italian woman, Giovanna, 10 years his senior. Although we held the wedding at our house, we and others in the lab, advised against the marriage. We did not think they were suitable, not only because of the age difference, but also in character. Initially everything seemed fine, Raniero moved back to Rome on the urging of his wife, two children were born, and he found a job in cosmetics (regulatory affairs) with Proctor & Gamble. This worked required living in England, which Giovanna did not like and after ups and downs they divorced. His-wife moved to California with the children, and Raniero remained in England. He then worked for L’Oréal, and now works for Estee Lauder is an expert cosmetic regulatory and safety, is

happily remarried and has two more children. He basically has found a niche in the cosmetic business, as an expert on Regulation and Safety.

Gensheng Feng and IDO

During this period two Chinese "visiting scholars" from Hangzhou University joined the lab. There existed an exchange program between Hangzhou University and Indiana University. I was to visit Hangzhou University a few years later. Both Dr. Ma and Dr. Din recommended one of their students Gensheng Feng as a potential graduate student. They considered him brilliant, although he came from a "peasant "background. He had left his village and family (probably illiterate) and moved to the city or was selected by the communist party because of outstanding grades in high school to study at university.

I remember picking Gensheng up at the airport. His English was just passable, with a peculiar "English "vocabulary. I think he referred to me as 'old fellow" and used dated colloquialisms. We had him over for a meal the next day, and taught him to use a knife and fork, and general "American" manners. He certainly caught on very fast. He arrived in the category of "visiting scholar" which was not the same as a graduate student. He arrived by himself, but showed me photos of a very pretty girl, whom one day he hoped would be his wife. It did not take long for him to assimilate into the lab and his surroundings, although again I had to teach him the "American way" of behaving. He expressed a wish to work on interferon, which he had previously done in China. We decided to work on two independent projects involving interferon-gamma. Mike Shepherd of Genentech had constructed a plasmid that contained both the interferon-gamma and tumor necrosis factor (TNF-b) gene. It was hoped that this plasmid construct, which could be grown in E. coli, would have the activity of both interferon-gamma and TNF and be used in cancer treatment. Although the product did work in cell

culture and quickly destroyed tumor cells TNF proved to be very toxic in vivo in mice. There was evidence for the production of a hybrid protein. With Mike Shepherd and a coworker from Genentech who constructed the plasmid, we published a paper in Science. However, our data indicated this combination would not be suitable for human clinical trials.

The second project, which was to give far reaching results, was to isolate cells that would be genetically resistant to either interferon-gamma, tumor necrosis factor or both. We decided to use the cell line ME180, of human cervical cancer origin, since it appeared to be uniquely sensitive to both cytokines. (This cell line may be an offspring of HeLa or a contaminant of the same). Gensheng treated the cells with a well-known mutagen, and isolated colonies (clones) of cells that grew in the presence of either interferon or TNF-b. Although we isolated cells resistant to both "drugs" we decided to concentrate our research on resistance to interferon-gamma. These mutants allowed us to separate the anti-viral function from the anti-proliferative function of interferon, since interferon treatment of the cells still prevented virus (herpes) growth. We then searched the literature for a clue as to the mechanism of this resistance. I discovered, what I now consider a classic paper, a 1984 publication by Elmer Pfefferkorn describing how interferon-gamma blocked the growth of the intracellular parasite toxoplasma gondii by inducing the breakdown of the essential amino acid tryptophan. This was through the activation of an enzyme indole amine 2,3 dioxygenase (IDO). We hypothesized that our mutants were possibly defective in this enzyme and thus were resistant to interferon. This was confirmed by direct enzyme assays, showing that the two mutants examined had decreased amounts of IDO. We then decided to do a thorough literature search on IDO, discover what was known about the enzyme, its history, and possible relationship to disease and tryptophan metabolism. This

review was co-authored by Gensheng Feng and myself in FASEB (Federation of American Societies of Experimental Biology) journal, and to date it my most cited paper. It was an excellent all-encompassing review. Through this work we became recognized as an IDO lab and had communications with other labs working in the same area, both in the US and in Japan.

It still surprises me to this day how much trouble I had in obtaining Gensheng's admission into graduate school, and later the completion of his Ph.D. One of our faculty objected to the automatic admission of a "visiting " scholar, and perhaps correctly insisted that any such student should take his class and sit exams. This was a difficult class in which many graduate students once admitted avoided or received poor grades. Gensheng took the class and proved his worth, there were no problems, other than one of language. Unfortunately, he never was able to pass the English exam for foreigners, so that we had to make an exception and allow him to teach without passing this exam. He taught my virology lab course as an assistant and was voted by the students an outstanding teaching assistant. Gensheng went on to contribute to other papers on the subject of regulation of IDO. He received his Ph.D. in 1990 and following a stay in Toronto as a post-doc he took a faculty position at IU Medical School. He is currently a full professor at UC La Jolla (San Diego). He is undoubtedly one of my most successful students. Dr Ma and Dr Du proved to be good judges of character.

Amgen and consensus interferon

I went to the annual interferon meeting in Florence, Italy in October 1990. Usually one goes to meetings to meet colleagues, listen to a few papers, present some work (in15 minutes), and enjoy the sights and restaurants of the location. The Interferon and Cytokine society rotate the meeting among continents, usually in great places (as do most scientific society) as a way of encouraging participation.

In 2010 it was again in Florence, in 2012 in Geneva, and in previous years in Vienna, Bologna, Montreal, Jerusalem etc. At this particular meeting in 1990 I met a student who had been a student in my virology lab class, and also had worked one summer in the lab of Stephen Surzycki during his undergraduate days. He had graduated sometime in the late 1980's. He introduced himself during a cocktail party (all these meetings begin with a very lavish cocktail party, usually thrown by the host city), his name Larry Blatt. He was a very outgoing person, and we talked a little about his past. After graduation he had worked for a time in biotechnology at Monsanto in St. Louis and then moved to Amgen, at Thousand Oaks, California. He suggested we have dinner one night together since he wanted to discuss some work and possible collaboration.

Amgen was one of the new biotech companies and had established itself quite early by patenting the cloning and production of Erythropoietin (epogen) a molecule used in enhancing growth of red blood cells, used in the treatment of patients on kidney dialysis and recovering from cancer chemotherapy. At that time this drug was the mainstay of the company, although since then other companies have produced variants of the drug, and the effectiveness of epogen itself has become controversial. Amgen has also developed recombinant drugs mostly involved in maintenance of red and white blood cells, and TNF receptors that block the activity of TNF. Larry had been working a short time at Amgen, and in looking for a project (that was the way Amgen worked in the early days) he came across an interferon, labeled consensus interferon, that had been created as a "theoretical " molecule containing the amino acid sequences of the most common alpha interferons (there are probably about a dozen of these). The story is that a group of Amgen workers, one evening over beer, decided to perform evolution in the test tube constructing the perfect molecule. Thus, consensus interferon was

born. The production of the consensus interferon is described by Feischko and Ritch (1986), (Chemical Engineering Communications). Initial data indicated that this interferon had high anti-viral activity. However, this interferon had not been tested thoroughly in vitro (for anti-viral or anti-proliferative response) or for in vivo activity. Larry suggested that we compare its activity with that of other commercially available alpha and beta interferons, for both activities, and I agreed. Amgen initially supported this research for a small sum of money. From this started an ongoing relationship both scientific and personal with Larry and continued with support from Amgen for many years even after Larry left the company. Unfortunately, Amgen lost interest in the consensus interferon and licenses it to a Japanese company, Yamamouchi in 1996.

Shortly after that meeting, a new post-doc from Israel joined the lab. He was like a whirlwind of activity. Zvi Reiter had come from Menachem Rubinstein's lab at the Weizmann Institute in Israel. He was very ambitious and wanted a hand in everything taking place in the lab. His aim appeared to be to produce as many papers as possible, even if the work was repetitious, and even if I doubted the importance of the publication. His enthusiasm often carried him away, and later led to clashes with Larry. He resented the fact that we were doing work for industry, that Amgen reaped the profits even though he himself was supported by a grant from Amgen. This was reminiscent of the old socialist ideology of no work for the capitalists! He was willing to bite the hand that fed him. On return to Israel, after about a year he dropped out of science and became a high school teacher, and later a principle in a prestigious high school.

We must have started work with the consensus interferon (later called infergen) shortly after the meeting in Florence, characterizing its activity and comparing it with the activity of commercially available interferons made by other

companies: Intron-a from Schering and Roferon from Roche. We indeed found that it had 10-fold higher antiviral and anti-proliferative activity compared to other commercially available Interferons. This first paper on its biological activity has been often cited (237 citations) and this work was the basis for clinical trials later on. Consensus interferon has been used for treatment of AIDS and hepatitis when other interferons have failed. It is still used in the treatment of hepatitis C although will probably be replaced by nucleoside analog inhibitors.

I note from the authorship of this first paper that I had a completely new crew working on interferon. Susan Klein, a biophysicist arrived in Bloomington because the physics department hired her husband. She joined the lab in 1990 and was a mainstay for a number of years. She lived in the country a few miles from Bloomington and her house became the venue for great New Year and other lab parties. Her golden retriever became the lab dog, and she welcomed the families of all the foreign students joining the lab. Her approach to research was very quantitative . Another author on this paper was Osman Ozes, a graduate student from Turkey, who completed his Ph.D. working on the biology of IDO, but also contributed greatly to the work on the consensus interferon. Osman later returned to the lab after a few years as a post-doc for a short period. He then worked at Intermune when Larry was director of research at that company. His son, Ali, whom we all remembered as very “wild " kid graduated from the biology department with the Ph.D. in 2016 a very nice young man. Osman later started his own biotech company in Turkey then moved to California and Ali is back working under the tutelage of Larry at Johnson and Johnson.

One of the techniques that Zvi Reiter taught me (and the lab) was the isolation of a specific class of immune cells known as NK cells (Natural Killer cells). These are a type of lymphocyte that non-specifically destroy virus infected

cells and tumor cells. They are activated against their target by exposure to interferon, and in turn produce other types of interferons. We examined the effect of consensus interferon on NK cells and found enhanced activation compared to the other commercially available alpha interferons.

For the last few years, I myself had not really been working at the bench. The group was so large (at least 20 people) and the need for money to support so many technicians, post-docs and graduate students was so great, that I devoted my time to writing grants and papers. Since most of the lab researchers were foreign, I wrote the majority of the papers. Although someone like Zvi Reiter, and Osman Ozes could write English, their manuscripts still needed considerable editing. Most of the Chinese students had a very rudimentary knowledge of English and I had to completely write up their work.

Work on genetics of the interferon system continued, with Gensheng Feng isolating new mutants resistant to interferon-gamma. This was done in collaboration with a group in Austria, and in due course I visited their lab in Innsbruck. Zvi Reiter pursued the effects of various drugs on NK cell activity, and published the work in minor journals, some of which did not last more than a few years. The number of projects going on in the lab overwhelmed me. I had received funding from NIH for work on IDO, from Cell Genesis for work on adenovirus vector and gene therapy, and by 1992 I was receiving about $100,000 per year from Amgen to continue the work on consensus interferon. I used most of this to support post-doctoral fellows and graduate students and bought new equipment.

In 1993, when it was obvious that the consensus IFN had great clinical potential for the treatment of hepatitis C Larry asked me to go with him on a lecture tour of Japan. This was to explain the basic biology of the consensus IFN and to “sell it” to Japanese physicians, since hepatitis C was a large problem in

Japan (and also in China). Hepatitis C is caused by a virus, HCV, and still is the leading cause of liver disease, and liver cancer. Until recently the only treatment has been interferon, and a combination of interferon and an anti-viral drug ribavirin. However, the rate of response to this combination is only about 40 %. At the time that this trip was made, only interferon was being used and the response rate was approximately 20 %. The normal course of treatment lasts 48 weeks, was accompanied by severe side effects, and was expensive. It was hoped that the consensus interferon would be better, and for some cases of HCV it is the drug of choice.

This was not my first time in Japan, and I enjoyed going to this country very much. I have written something of this trip and also a subsequent trip to China in a separate chapter.

We continued work on the consensus interferon, studying the kinetics of action, host range, receptor binding and many other aspects. Meanwhile it had entered the clinic and was being used as an alternative to Roferon and Intron, with similar or better response.

Meanwhile the work on IDO, its structure and biological role continued. After Gensheng Feng had finished his Ph.D., Koaukou Vincent Konan, a student from the Ivory Coast, continued this project. Vincent, as we called him, was an unusual graduate student. His native language was French, and he arrived with a poor knowledge of English. Although he had an undergraduate degree from a university in the Ivory Coast, his knowledge of biology, and in particular biochemistry was very limited. Initially the other students in the lab were impatient with him, and really had no time for his naïve technical questions. However, he learned very quickly, and for his MA thesis project decided to work on APRT/ adenovirus vector. After his MA, which resulted in two publications with other

students, he decided to work on the sequencing and regulation of the IDO gene. He very successfully cloned the gene and identified the promoter region (that region involved in regulation of the gene). IDO continues to this day to be of interest to immunologists, since there appears to be a relationship between T-cell tolerance and tryptophan degradation, and it has been suggested that IDO plays a role both in interferon induced depression, and in maintenance of the embryo during pregnancy. The effect on depression would be due to alterations in the levels of brain tryptophan and serotonin. At this stage our work on IDO stopped, since the grant proposal to continue this work was not funded, and my interests on interferon moved in another direction. Larry Blatt had by this time left Amgen, but I continued to receive support for work on consensus interferon.

Following a seminar given at the University of Illinois Medical School on consensus interferon, I met with Don Jensen and Scott Cotler, both renowned hepatologists. Since I was interested in the mechanism of consensus interferon and its effects on other cytokines (a type of hormone like molecule that acts on different cells of the immune system) we collaborated and measured the induction of other cytokines after initiation of treatment. Samples of white blood cells were shipped to us from Chicago and we measured cytokine production using commercially available ELISA kits. This is a kit in which an antibody to a substance is linked to a substrate, so that if an antigen (in this case a specific cytokine) interacts with the substrate a color reaction occurs. We found that IL6 is induced within a few hours of treatment with interferon and then declines within 12 hours. Other cytokines measured did not show any change within the time measured. The response of IL6 was very fast, within 4-6 hours, and correlated with the increase in fever and other side effects in the treated patients. Similar results were found with the IL1ra (a receptor analog for IL-1). This cytokine or cytokine

inhibitor has previously been shown to be induced by interferon. There was no difference found between hepatitis C patients who responded to interferon treatment and those who did not nor in a group of African American patients compared to Caucasian patients. Thus, we could conclude that all patients responded to interferon irrespective of early viral response. This implied that interferon interacted with its receptor irrespective of the state of the patient. Another interesting clinical observation reported around this time (1999) by an independent group was that African Americans patients had a lower response rate to interferon treatment than Caucasians. A proposal to examine this was funded by Amgen, and support continued even after Larry left the company.

By this time Amgen was no longer interested in consensus interferon and had sold the rights to a Japanese company (Yamanuchi) but retaining the American rights. It was obvious to Larry that Amgen had lost interest in the commercialization of the consensus interferon. Subsequently this was sold to a new biotech company, Intermune. Larry Blatt left Amgen, spent a short period at National Genetics Institute, a private hepatitis C testing company, and then moved to Intermune, which purchased the USA rights to consensus interferon and also was involved in the commercialization of gamma interferon. It is difficult to understand why Amgen abandoned its interferon program. It did have clinical potential. The CEO of Amgen wanted to pursue novel biochemicals and interferon did not fall into this class of molecule. Intermune has recently been purchased by Roche for 8 billion dollars. This was not on the basis of interferon but on another drug perfinidone that Osman Ozes did most of the ground work. This drug failed in the first clinical trials but was successful in a second trial.

Virahep C clinical trial.

I was still interested in the research initiated with Drs. Cotler and Jensen, whether other cytokines were induced (or regulated) by interferon and submitted a grant proposal to continue this investigation, using blood samples from patients with HCV, obtained from Dr. Paul Kwo, a hepatologist at the IU Medical School. This grant proposal was rejected.

While perusing a publication from the NIH I came across a request for proposals (RFP) that was very similar to the grant proposal just rejected. It was a request to establish a consortium of clinical and basic laboratories (called ancillary laboratories) to investigate the effect of interferon at the gene level (among other items) in hepatitis C patients. The main thrust of the proposal was to investigate whether reported differences in response rates between Caucasian and African American, was correct and whether the basis for this difference was in gene expression or selection for virus resistant to treatment. This was similar to work done with the consensus interferon.

I called Eli Ehrenfeld, the head of the NIH grants division to discuss the strategy to pursue, and she suggested I make minor modifications to the rejected proposal and resubmit. I rewrote my proposal in lines with the RFP and it was assigned to a special grant committee. To my surprise the proposal received a high score and was funded.

The outcome was a “consortium of laboratories to work on the differential response of African Americans and Caucasians to interferon/ribavirin treatment in the case of hepatitis C”. There were 8 clinical laboratories, where patient blood samples would be collected and 4 basic laboratories. each one studying a different aspect of treatment. My lab was initially consigned to look at interferon signaling, in other words to examine the spectrum of genes induced by interferon, with initial

emphasis on other cytokines. John Tavis (St Louis) was to examine the effect of interferon/ribavirin treatment on virus and whether the virus sequence differed in AA from CA patients, Leland Yee (Yang and group, Philadelphia) to analyze genetic polymorphisms in genes that were induced: and Hugo Rose (Washington-later Denver) to analyze the immune response in patient samples. The members of the consortium, met once every three months to plan experiments. This was the first time I worked in a group, and found it stimulating. The fact that we met routinely and were expected to present data certainly helped the work along. At each meeting I had to make a presentation (as did others) of progress, plans for the future, and problems. We could also propose other projects along the way, which were discussed by an ancillary committee. Most meeting were taken up by clinical problems, adverse effects of the treatment, how to deal with patient drop out, and discussion of the optimum statistical methods to use for analysis. Initially I had intended to just examine the response of a few genes, and to construct DNA microarrays for that purpose. After a few tries at constructing our own arrays, I realized how difficult it was, and how non-reproducible were the results, that I decided to look at alternatives. I learned that the Human Genome Center in Indianapolis, at the IU Medical School, already performed DNA microarray analysis using commercially available chips. I discussed the research with Dr. Howard Edenberg the director of the institute and reached an agreement. We would purchase the chips and pay for some labor costs, and they would allow us to tap into their software system for analyzing the data. Statistical help was provided by Dr. Jeanette McClintock of that group. We would receive the blood samples from the various hospitals and separate out the white blood cells, extract the RNA, clean it up on mini-columns and send it to Indianapolis for micro-array analysis. Later, the RNA extraction was handled by a commercial company, we cleaned up the RNA and sent it to Indianapolis. Since we were receiving samples from 90

patients, at different time points, there was an appreciable amount of bookkeeping, which was performed by my lab technician Mary Ferris. I spent hours (days, weeks) analyzing Excel spread sheets with the data from 20,000 genes from 70 patients (some samples were discarded) at different time points. The data were initially blinded so that I did not know the response or ethnic origin of the patients. This information was provided later for the final analysis. I must admit I learned a fantastic amount of statistics and employed a couple of people (William Grosse and Takuma Tsukahara) to help with the analysis.

At the same time, we exploited both the availability of funds and access to the Affymetrix microarrays system to analyze the effect of interferon on gene induction on cells in culture, comparing gene induction after treatment with IFN-alpha, IFN-gamma and both together, examining the synergistic effect. For this analysis I collaborated with a statistician in Israel (Haifa University), Leonid Brodsky, up until my retirement in 2008. We also performed similar work for Intermune (and Larry Blatt) on gene induction in liver cell lines in vitro with the consensus interferon - infergen and with pegylated gamma interferon.

Our preliminary work, once I had received the grant and was able to hire a post-doc and technician was to study the effect of interferon on peripheral blood monocytes (white blood cells) incubated with interferon for either 4 or 24 hours. This was to test the system, and to obtain experience of using the "portal" as it was called in Indianapolis, and to determine genes induced, and to examine whether genes known to be induced by interferon were detectable in this system. The first paper describing this work is, in my opinion one of the most thorough papers analyzing the hundreds of genes induced by interferon, and the relationships between and among these genes. It has been used in building what has been called the interferome, web site for searching tissue specificity of genes, etc.

Receiving this grant allowed me to hire a number of people. Among them was Corneliu Sanda, a young physician from Romania. He and his wife Alina were a delightful couple and because of Mimi's Romanian background we became good friends. They also became our guides when we visited Bucharest in the summer of 2003 or 2004. They showed us around the city, bought tickets to the National Opera, and introduced us to some good restaurants. Corneliu really was not all that interested in continuing in research, decided to go back to medical school, and is now a practicing physician in the area of drug rehabilitation, specializing in psychiatry. Alina is an infectious disease clinician. Both of them live in New York

The story of Bill Grosse is rather a sad one. He applied for a position in bioinformatics. I called his references and got positive feedback, except one of them mentioned that in the past he had some personal problems. Despite this I decided to hire him, he came across as very knowledgeable with extensive experience in microarray analysis. It was obvious after a short time that he was an alcoholic. He had been married was divorced, and had one child, a son, of whom he was very fond. His parents lived in Zionsville, but I gathered the relationship was not good. I first became aware of his problem when he and a student (undergraduate working in the lab) attended an American Society of Microbiology meeting at McCormick's Creek State Park. He disappeared in the evening. Not attending the session, he took the undergraduate with him, and went to a local Karaoke bar. He got very drunk and was helped back to his room by the student, who was in a state of shock, never having experienced this type of behavior before. He was supposed to return to Bloomington the next day with Bill, who was so inebriated that he was unable to drive back, and I had to make alternative arrangements. Bill then brought to Bloomington a "lady " friend a body builder

who was tattooed from head to toe. She was from somewhere in Pennsylvania and had five children. I assume she left them with her ex-husband. Shortly after her arrival in Bloomington they were wed, despite the active opposition of Bill's parents. Within a few weeks there was obvious trouble. She was ordered back to Pennsylvania to look after the children (I do not know the details), and he told me that he would have to look for another job in that State in order to follow her. He left shortly for Pennsylvania, and then I believe moved to St Louis, I assume without her. In September 2005 I received a phone call from a lawyer asking me if I had any property or papers belonging to Bill Grosse. I was surprised and asked what the problem was. He informed me that Bill had died from binge drinking a few days before. He was 41 years old. What a waste of a talented life.

The clinical trials on this project continued for approximately 5 years. We had meetings in Washington (Bethesda) or at other clinical sites throughout this time, and we built a very good relationship with both the people in the other ancillary labs and other investigators. The general idea was that we would identify genes whose expression was altered by interferon treatment and analyze whether the level of gene expression differed between those patients responding to treatment and those not, or those partially responding. A second aim was to identify differences in gene expression between African American patients and Caucasian patients. Leland Yee and his group in Philadelphia would examine genetic differences in these particular genes. John Tavis, University of St Louis, examined changes in virus sequence in these specific patients and Hugo Rosen looked at the immune response in vitro in lymphocytes from these patients. We accrued masses of data. We performed DNA microarrays on 69 patients, at multiple time points during the first few months of treatment and examined some

20,000 genes. We found that some 1000 genes either had increased expression, were turned on, or had decreased levels of expression after treatment, and these changes were observed within 24 hours of the first injection of interferon. Many of these were genes previously identified by others as being related to the interferon response. Our list of genes was the most comprehensive ever produced. Although we found that levels of gene expression correlated with response, this appeared to be global, and not due to any specific gene. We also were unable to identify differences between African Americans and Caucasians. This may have been due to using blood cells (lymphocytes) rather than liver tissue, but there was no way we could justify doing liver biopsies every few days. There were many variables that might have affected our work, including the collecting of blood at different centers, the shipping to another center for RNA extraction which often took a few days, and the conditions in which the RNA was held in that laboratory before shipping to us. We processed the RNA further to clean it up, and it was shipped again (or delivered directly) to Indianapolis for array analysis. We (I and some statisticians) received the data for further analysis. After the departure of Bill Grosse, I hired a very good MS student from Informatics Takuma Tsukahara, and together we did most of the analysis.

This ViraHep C project resulted in many publications, some collaborative with the other laboratories involved, and some just from my lab. At least 14 papers were published from my laboratory, and another 4-6 as a result of collaborations without my name as an author,

As a result of this work, representatives of Schering approached me and requested that we do a similar analysis with patients treated with their interferon (Peg-Intron A). Their scientists believed that their interferon was superior to that used in the above clinical trial, which had been supplied by Hoffman-Roche

(Pegasys). In collaboration with Dr. Paul Kwo we mimicked the protocol used in the virahep C trial, but used different time points, and the interferon and ribavirin were administered on the bases of body mass, rather than a standard dosage. Twenty patients were enrolled, without race being a factor, and similar DNA microarray analysis performed. We again could not find any major differences between those patients that responded to treatment and those who did not, and the values (fold induction) were very similar to the previous trial. As in the previous trial the majority of genes were induced early, with return to normal for many genes within a few weeks of treatment. The data was disappointing both to me and to the scientists at Schering.

Work being done at other laboratories where liver was used for analysis rather than blood, indicated differences between responders and non-responders, but not as expected. Non-responders appeared to have higher levels of interferon induced genes before treatment initiation, and this was only in a subclass of liver cells. Thus, when the grant period finished the situation was murky and still is. However, treatment with interferon seems to be dying out. New drugs, protease inhibitors and nucleoside inhibitors are now entering the market. Larry Blatt who in a way was responsible for my venture into the world of consensus interferon is now CEO of a company, Alios that is developing some of these new anti-hepatitis C and anti-viral drugs, one of which is in phase 2 trial. Alios was sold in 2014 to Johnson and Johnson for 1.5 billion dollars.

I have continued to use the data generated by the microarrays, and in collaboration with a group at Yale have published some of the unpublished data. The data was also used by an informatics student, Rahul Gupta, to complete his

MS degree in informatics. I will not be surprised if the data continues to be used in the future. It was on this note that I basically ended my scientific research career.

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