GENTIAN VIOLET IN BLOOD BANKS, THE CURE OF CANCER, AND THE Y STRAIN OF TRYPANOSOMA CRUZI.

Recollections of Ruth and Victor Nussenzweig

(both MD.PhD, Professors at New York University School of Medicine)

Ruth and I are happy to describe the very beginning of our scientific careers. It was really lots of fun**:** we had no deadline to meet, no thesis to complete, no prelims, practically no advisors, and doing mainly research which we thought could be useful, what is now known as "translational research".

What we will tell you involves two entirely different projects that Ruth and I started during our last two years of Medical School. Both projects were very ambitious: the first dealt with the possibility of killing *Trypanosoma cruzi* in blood destined for transfusion. The other was an attempt to reproduce published results by two Soviet investigators who claimed that the *T. cruzi* extracts could treat certain types of cancers. The only link between the two projects was that both required the isolation of a virulent strain of

T. cruzi highly infective for mice.

We entered medical school in 1947, after high school, when we were 18 years old (no college in Brazil). A few years earlier, shortly after the end of the Second World War in 1945, Ruth had escaped from Vienna where she was born. At that time, democratic ideals permeated Brazilian society and the dictatorship of Getulio Vargas ended. For the first time in 15 years, free elections were held with the participation of several political parties. During our first two years in Medical School, Victor did not contemplate a career in science. He joined a group of leftist students that wanted to combat the obvious social inequalities in Brasil. In the third year he started dating Ruth, who was wiser. She convinced him that he might make a much greater contribution to Brazilian society by becoming a scientist, than by attending boring, and mostly useless political meetings.

At that time we read a sensational article by two Soviet scientists, Professors Ruskin and Klueva.

They claimed that *T. cruzi* extracts inhibited the growth of cancer in some patients, and that *T. cruzi* infection did shrink certain transplantable tumors in mice. A well-known group of scientists from the National Cancer Institute in the US had confirmed the effect of live parasites on certain cancer of rodents. The findings of Ruskin and Klueva were widely reported. In fact, many years later we found that Cruzin, the Ruskin/ Klueva *T. cruzi* extract, was still sold in certain drugstores in Paris! The French are conservative, and may be Cruzin is still available there.

We decided to discuss the Russian's claims with Samuel Pessoa, the Parasitology chairman of our Medical School, an internationally renowned Public Health scientist. He was loved by students**:** he gave very funny lectures, was socially minded, and had a very upbeat personality. When we told him that we might cure cancer with a parasite, he told us that this was a fantastic idea. Surely he knew that our project was crazy, and that we had absolutely no laboratory experience. Nevertheless, he immediately provided us with space, and the required equipment (a microscope and a small centrifuge) and supplies. To this day, we are grateful to him.

The "space" was a large empty laboratory that belonged to an Associate Professor (Dr. J.L.P. Freitas) of his department who worked on *T. cruzi*.

However, no highly virulent *T. cruzi* strains that could be maintained by passage in mice, were available at that time in Brazil. It took some months to isolate *T. cruzi* from the feces of Triatoma bugs that had fed on a woman suspected to have chronic Chagas disease. Ruth and I were so appalled with the xenodiagnostic procedure (live bugs, as large as roaches, feeding in patients) that we decided to feed the bugs on drawn blood *in vitro*. This worked, and the Departmental Chair, not only encouraged us to publish these findings, but included the description of the "*in vitro* xenodiagnosis" in his textbook of Parasitology. He certainly knew how important it is to feed the ego of budding scientists!

Many years later, during a visit to the Weissmann Institute, we met a Soviet scientist who told us that Ruskin and Klueva got in serious trouble in the Soviet Union in the late 1940's. Both of them, together with their direct supervisor, were asked to attend a special meeting of the Central Committee of the Communist Party. In the presence of Stalin, they were severely admonished for having disclosed a state secret to American scientists! Their careers ended, and their supervisor was sent to a labor camp in Siberia.

After multiple needle passages in mice (with no gloves!), the newly isolated *T. cruzi* strain had the required properties. The parasitemias were consistently high, and the mice died during the acute infection. We then obtained a transplantable tumor from another Research Institute, in Sao Paulo. We set up experiments to measure the tumor growth in *T. cruzi* -infected and non-infected outbred mice. No inbred strains of mice were available in Sao Paulo, nor elsewhere in Brazil, at the time. The growth of the tumor in the controls was so variable that it was impossible to reach any conclusions-- so the second project was a failure.

The Y strain of *T. cruzi*, (initial of the patient's name) that we had isolated has since been widely used in Brazil and abroad. The manuscript describing its properties, is widely quoted (but

most likely not read). It was co-authored by a colleague and close friend of ours, Luiz Hildebrando Pereira da Silva, who later went on to become head of the Parasitology Department of the Pasteur Institute, in Paris. He is now back in Brazil, in the Amazon region, training as well as directing a malaria research group and others working on local diseases, and writing his memoirs.

Our third project dealt with the transmission of Chagas Disease by blood transfusion, and its prevention. An important figure in this story was Dr. Freitas, who had developed a more precise diagnostic tools for this disease based on the fixation of complement by antibodies in the patients serum. For a while, during medical school, Victor was in charge of performing routinely this assay in the University Hospital. This is how Victor developed an interest in basic complementology, a subject he later pursued for many years.

One day in 1951 Dr. Freitas held a meeting with us and two colleagues, a young resident in infectious diseases and a blood bank physician. He proposed to us a project to determine whether Chagas disease could be transmitted by transfusing blood from chronic carriers of this disease. In these patients the parasite is extremely rare. Although transmission by transfusion was likely, and widely discussed, a definitive answer was not available. Dr. Freitas suggested that we perform the new optimized serological test in a large group of blood donors, and follow the recipients patients who had received serologically positive blood. Ethical concerns were discussed during that meeting. But blood donors had never before been screened serologically for Chagas disease, neither at our University's hospital, nor anywhere else. The blood would be transfused without knowing the results of the assay, and we were not sure that transmission would indeed occur. It was decided to proceed. It is unlikely, however, that this project would nowadays be approved by the ethics committee of any hospital!

The outcome of this trial was that 3 out of 13 transfusion recipients became infected by *T. cruzi*. This was particularly frightening because, at that time, in some endemic areas of Brazil and other South and Central American countries, more than 20% of all blood donors were estimated to be at a chronic inapparent stage of Chagas' disease. Moreover, blood banks did not perform any serological tests to exclude these donors.

There was another meeting in which two additional topics of investigation were raised**:** to determine the prevalence of chronic inapparent Chagas disease among blood donors in various blood banks, and whether it would be feasible to add trypanocydal drugs to the blood prior to transfusion and thus prevent disease transmission. Ruth and I were asked to deal with the latter question, and we accepted the challenge. Dr. Freitas must have realized the difficulties of this project, but thought that it was appropriate for crazy medical students who had tried to cure cancer.

By that time our relationship with Dr. Freitas had soured. We did not ask for his advice, nor discuss our research with him. We now believe that he had good reasons to be upset. Victor was quite arrogant, and our laboratory was very messy, with mouse cages, racks of used tubes, notebooks, etc. scattered all over the place. In sharp contrast, Dr. Freitas was extremely well organized, and his laboratory a model of neatness. Aggravating the situation the chairman of the

Department had given us space that belonged to him!

Our approach to find a trypanocidal drugs was simply to add different drugs to samples of heavily infected mouse blood infected with the Y strain, and examine the blood under the light microscope to observe the *T. cruzi* parasite motility. If abolished, we injected the treated blood sample into naïve mice to be certain that those highly virulent parasites were no longer infective. It was a time of suspense, one experiment a day! We went to the laboratory, clipped the tails of the mice we had infected with blood containing immobilized *T. cruzi* and examined it under the microscope.

After a few failures we decided to try dyes. The main reason was that we had read Paul de Kruif's book "The Microbe Hunters" (we highly recommend it). We were impressed with Paul Erlich's use of dyes for chemotherapy. The question was which dyes to try? Dr. Michel Rabinovitch at that time working in the Department of Histology and Embryology of our Medical School (later on at the Rockefeller University) gave us a text book listing the dyes available for staining tissue sections.

Our first choice was gentian violet, because we knew that humans afflicted with severe infections by *Strongyloidis stercoralis*, were treated by intravenous injections of this dye, without deleterious side effects. This information was in the Parasitology textbook written by our chairman, and we had to read it thoroughly since both of us were laboratory instructors.

To our delight, the first experiment showed that gentian violet inhibited the motility and abolished the infectivity of *T. cruzi* trypomastigotes. We determined that the amounts of gentian violet needed to kill the parasites in one unit of blood were routinely used for the intravenous treatment of several worm infections. These experiments were done in collaboration with Ms. Judith Kloetzel who received a small fellowship from the owner of a publishing empire in Brazil. Several years later our friend Judith got her PhD degree, continuing to contribute to Parasitology research.

We had one serious concern while performing these studies. The precise composition of the gentian violet was not given on the flask. We only knew from the Merck Index that gentian violet is a mixture of three dyes: crystal violet, methyl violet, and brilliant green. We wrote a letter to Eli Lilly, the manufacturer, inquiring about the precise composition of their product. In it we presented our results (no thoughts of patent protection), explaining the importance of the problem. After several months the response came by a non-signed letter from the company, stating that the composition of gentian violet was a business secret. Years later Judith determined that crystal violet alone was as effective as gentian violet. By that time gentian violet had already been added to hundreds of transfusions, without any ill effects, except that the blood had a bluish tint.

The remaining question was whether gentian violet was in fact effective. We did not want to repeat the study that had revealed the potential of blood transmission of this disease by blood coming from known sero-positive blood donors. The ethical concerns were obviously too great. This was resolved when one member of the team who needed frequent blood transfusions

(neither of us), transfused himself with 420 ml of blood from a patient in the acute stage of Chagas disease, that had circulating parasites detectable by light microscopy!! Before transfusion, gentian violet was added at a concentration of 0.5 g per liter, and the blood kept at 4ºC for 48h. This physician had witnessed our experiments, and had faith in gentian violet power. He was carefully monitored parasitologically and serologically. No infection ever developed.

New experiments were then done soon afterwards. Eighteen recipients of gentian violet –treated blood from chronic Chagas disease were closely monitored. To our great relief, none were infected. The results of our experiments were communicated in various meetings, and then published. Many blood banks in Chagas endemic areas of Brazil and elsewhere in Latin America, started to add routinely this dye to all blood packages. It did not take long for gentian violet-containing transfusion bags, manufactured in the US, to be distributed in Latin America. In a review published in 1990, more than 100,000 patients had received blood transfusions containing gentian violet, without side effects. The maximum amount of treated blood given to a single patient within six months was 68 units (34 liters),s and no toxic effects were observed. Most importantly, there has been no reported case of Chagas disease transmission among the very large number of recipients of gentian violet-treated blood. What is the mechanism of action of gentian violet? Drs. Roberto Docampo and S. Moreno, professors at the University of Illinois, showed that this cationic dye affects the function of the parasite's mitochondrion and depletes ATP.

Chagas disease is currently under control in Brazil. Spraying DDT on the walls of the mud houses kills the vector and to date Triatomes have not developed DDT resistance. Although acute Chagas is extremely rare in Brazil, a large number of carriers still remain. These can now be excluded as blood donors by epidemiological screening, and by sensitive serological tests available in most blood banks in Brazil.

However, transmission of infectious agents by blood transfusion remains a challenging problem. Blood banks have now to deal with the threats of inadvertent transmission of hepatitis B and C virus, HIV and West Nile Virus, and the possibility of sterilizing the blood with drugs is under investigation. We were happy to learn at a recent meeting dealing with blood transfusion, that our rather primitive studies performed during medical school while skipping most of our classes pioneered this field.