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RESEARCH ARTICLE

Correlation between viral load of cytomegalovirus and tacrolimus and sirolimus levels in transplanted pediatric patients

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KEYWORDS
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Cytomegalovirus;
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Abstract
Introduction: Survival of transplant patients and grafts depends largely on the use of immunosuppressive drugs. However, a balance remains to be established among immunosuppression, transplant rejection and cytomegalovirus (CMV) infection, which results in a high rate of morbidity and mortality. The aim of this study was to define a better strategy for monitoring transplanted patients based on the analysis of the blood concentration of sirolimus and tacrolimus and the burden of CMV.
Methods: Fifty five post-transplant (kidney and liver) pediatric patients, nine treated with sirolimus and 46 treated with tacrolimus, were included. A total of 541 measurements were obtained. In each measurement the concentration of immunosuppressant in whole blood and CMV viral load in plasma and whole blood was quantified by real-time PCR. Pearson correlation coefficient (r) was estimated.
Results: Values of $r \leq 0.0747$ were found for the relationship between dose and concentration of immunosuppressant; $r = 0.9406$ for the relationship between viral load in whole blood and plasma, and $r \leq 0.4616$ for the relationship between concentration of immunosuppressant and viral load.

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Conclusions: These data support that the doses of immunosuppressive drugs do not correlate with the levels of the same in whole blood. Therefore, systemic levels of immunosuppressant should be constantly monitored together with CMV load. Meanwhile, a high correlation between viral load measured in whole blood and plasma was found.

PALABRAS CLAVE
Trasplante;
Citomegalovirus;
Sirolimus;
Tacrolimus

Correlación entre la carga viral de citomegalovirus y los niveles de tacrolimus y sirolimus en pacientes pediátricos trasplantados

Resumen
Introducción: La supervivencia de pacientes trasplantados y de los injertos depende en gran medida del uso de fármacos inmunosupresores. Sin embargo, aún no se ha logrado establecer un balance entre la inmunosupresión, el rechazo al trasplante y la infección por citomegalovirus (CMV), lo cual deriva en una alta tasa de morbilidad y mortalidad. El objetivo de este trabajo fue definir una mejor estrategia de seguimiento de los pacientes trasplantados a partir del análisis de la concentración en sangre de sirolimus y tacrolimus y la carga de CMV.
Métodos: Se incluyeron 55 pacientes pediátricos post-trasplante (riñón e hígado), nueve en tratamiento con sirolimus y 46 en tratamiento con tacrolimus. Se obtuvieron 541 mediciones en total. En cada medición se cuantificó la concentración de inmunosupresor en sangre total y la carga viral de CMV en plasma y sangre total mediante PCR en tiempo real. Se calculó el coeficiente de correlación de Pearson (r).
Resultados: Se encontraron valores de $r \leq 0.0747$ para la relación entre dosis y concentración del inmunosupresor; de $r = 0.9406$ para la relación de la carga viral entre suero y sangre total y de $r \leq 0.4616$ para la relación entre concentración de inmunosupresor y carga viral.
Conclusiones: Estos datos apoyan que la dosis de los fármacos inmunosupresores no correlaciona con los niveles de los mismos en sangre total. Por ello, deben ser constantemente monitoreados junto con la carga viral. Por su parte, se encontró alta correlación entre la carga viral medida en sangre total y plasma.

1. Introduction

Solid organ transplant (SOT) is a viable option for the treatment of childhood diseases that result in end organ failure. According to the Organ Procurement and Transplantation Network (OPTN) in the U.S. in 2008, almost 2000 pediatric patients (<18 years) received an organ transplant, representing 7.6% of all transplants performed. The majority of those transplants were kidney followed by liver. Among the principal problems associated with the transplant are graft rejection and infection due to cytomegalovirus (CMV).[1,2]

CMV or human herpesvirus 5 was initially isolated from the salivary glands and kidneys of children.[3] It received the name of cytomegalovirus because it produces typical cytomegalic inclusions in the affected cells.[4,5] In 1965 it was isolated in a kidney transplant recipient.[6] Infection caused by CMV is present in 90% of the world population, usually since childhood.[7] In patients who are immunocompromised, such as patients with SOT, CMV is one of the principal causes of morbidity and mortality because it causes clinical effects such as viral syndrome by CMV, fatigue, organ failure and graft rejection.[8] In addition, these effects are associated with an increase in hospitalization costs.[8–12]

Despite advances in the development of immunosuppressive agents, a balance between therapy to prevent rejection and, at the same time, preserve the ability of the immune system to control or prevent infectious processes has not been found. Two of the most commonly used immunosuppressants are tacrolimus and sirolimus. Currently, for management of patients with SOT, the best strategy to prevent rejection associated with insufficient concentrations of immunosuppressant and CMV disease is to individualize therapy. Preventing rejection is achieved by routinely quantifying the immunosuppressant levels in blood to keep them within the therapeutic range. In regard to CMV disease, prevention is carried out by monitoring the viral load in whole blood and/or plasma.[13–16] The solid organ transplant program of the Hospital Infantil de México Federico Gómez (HIMFG) is one of the main programs in Mexico. In that institution no studies have been carried out that document the quantitative relationship between the dose and the blood levels of the immunosupresant or that show the

relationship between the blood level of the immunosuppressant and CMV viral load in whole blood and in plasma. The goal of this investigation was to describe the correlation between the blood level of tacrolimus and sirolimus immunosuppressant agents and CMV viral load in pediatric patients with kidney or liver transplant from the HIMFG.

2. Methods

2.1. Study population

The study was conducted at the Clinical Laboratory Department of the HIMFG between May 2011 and June 2012. Pediatric patients with kidney or liver transplant who were undergoing immunosuppressant treatment with tacrolimus or sirolimus were included. The study included nine post-kidney transplant patients undergoing therapy with sirolimus as immunosuppressant. From these patients, 13 measurements were done. The study also included 46 patients undergoing treatment with tacrolimus as immunosuppressant (20 post-liver transplant and 26 post-kidney transplant). There were 528 measurements done from the group of patients undergoing therapy with tacrolimus.

The study was approved by the Scientific Research and Ethics Committees of the HIMFG. Throughout the study the identity of the patients remained confidential, although the series of clinical tests done is part of the routine follow-up performed after transplantation as a strategy to reduce the risk of CMV disease.

2.2. Quantification of sirolimus in whole blood

For quantification of sirolimus, pre-treatment using a 150-μl sample of whole blood and 300 μl precipitating reagent from ARCHITECT Sirolimus Whole Blood Precipitation Reagent® (Abbott Laboratories) was carried out. The mixture was incubated at 42 °C for 10 min. It was then centrifuged for 7 min at 13,000 rpm, and the supernatant was separated in a pretreatment tube for immunosuppressive techniques (1P06-01®, Abbott Laboratories). The mixture was centrifuged for 30 sec and finally quantified with the ARCHITECT *i1000SR*® (Abbott) equipment. The manufacturer's instructions were followed in all procedures. Quantification of the immunosuppressant was reported in ng/ml.

2.3. Quantification of tacrolimus in whole blood

For quantification of tacrolimus, pre-treatment was carried out by placing a sample of 200 μl whole blood and 200 μl of a precipitating reagent (ARCHITECT Tacrolimus Whole Blood Precipitation Reagent®, Abbott) in a centrifuge tube and then using vortex agitation for 7 min. It was then centrifuged for 7 min at 13,000 rpm, and the supernatant was separated in a pretreatment tube for immunosuppressive techniques (1P06-01®, Abbott Laboratories). The mixture was spun for 10 sec and quantified in the ARCHITECT *i1000SR*® (Abbott) equipment.

2.4. Quantification of CMV

Quantification of CMV was done in whole blood and plasma with real-time PCR. For this procedure, 3 ml of peripheral blood was obtained anticoagulated with EDTA. An aliquot of 400 μl was separated. The remaining sample was centrifuged at 3000 rpm for 10 min and an aliquot of 400 μl of plasma was taken. For extraction of the DNA the MagNA Pure Compact® (Roche Molecular Diagnostics) equipment was used with a set of reagents using MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Molecular Diagnostics). Programs for DNA extraction from 400 μl of peripheral blood were used with an emulsion volume of 200 μl, and the program of total nucleic acids from 400 μl of plasma with an emulsion volume of 100 μl. For real-time PCR a 166-bp fragment of the CMV viral genome was amplified a design from the TIB MOLBIOL Company and LightCycler® equipment (Roche Molecular Diagnostics). As internal positive control (human albumin), a set of primers that amplifies a 278-bp fragment was used. Primer sequences against CMV and the albumin gene are not specified by the supplier. The reaction mixture was brought to a final volume of 20 μl using 5 μl of the DNA extracted from each of the samples. The reaction mixtures were subjected to a program of denaturation of the sample and activation of the enzyme at a temperature of 95 °C for 10 min, followed by 50 amplification cycles of 5 sec at 95 °C, 10 sec at 60 °C, 15 sec at 72 °C. Later, a denaturation curve was performed to identify the product of the PCR derived from the DNA of the CMV, with a program of 1 cycle for 20 sec at 95 °C, 20 sec at 40 °C and 1 sec at 85 °C, with an increase in temperature of 0.2 °C sec and continuous fluorescence acquisition mode. The manufacturer's instructions were followed in all cases (Roche Molecular Diagnostics).

2.5. Statistical analysis

To evaluate the correlation between study variables, Pearson correlation coefficient (r) and its respective statistical value (two-tailed p value) were obtained; $p < 0.05$ was considered to be statistically significant. The statistical package GraphPad Prism v.5.0 for Windows (GraphPad Software, La Jolla CA, www.graphpad.com) was used.

3. Results

The study included nine patients with the immunosuppressant sirolimus and 46 patients with tacrolimus. The immunosuppressant dose varied from 1 to 3 mg every 24 h for sirolimus and from 0.25 to 6 mg every 12 h for tacrolimus. Figure 1 represents the correlation between the dose administered and the levels of immunosuppressant in the blood. There were 13 measurements done for sirolimus and 528 for tacrolimus. A positive correlation was found between variables in both cases, although the Pearson correlation coefficient (r) was very close to zero ($r = 0.0747$ for sirolimus and $r = 0.3037$ for tacrolimus). In the latter case, the correlation was statistically significant ($p = 0.0001$). However, this means that only with a 30% certainty could one predict the blood concentration of tacrolimus with respect to the dose administered. In relation to sirolimus, there was no correlation observed between the dose and the concentration

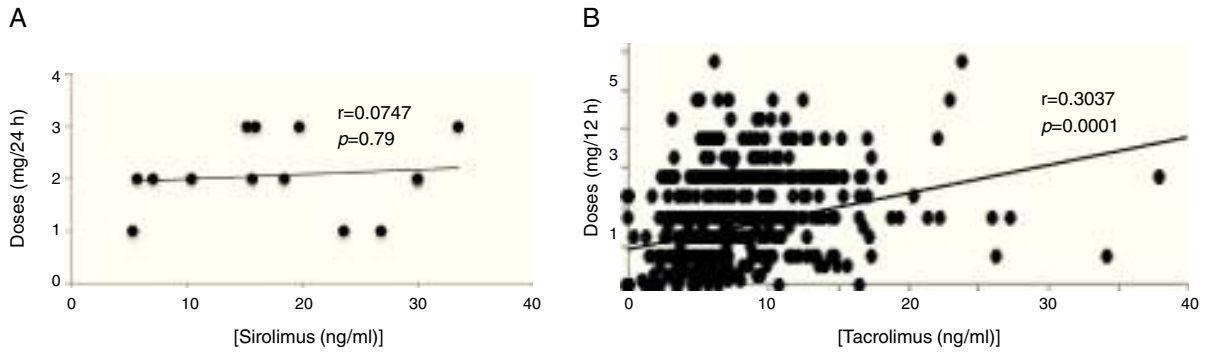

Figure 1 Comparison of dose vs. concentration in blood of the immunosuppressant. (A) Sirolimus (13 measurements from nine patients). (B) Tacrolimus (528 measurements from 46 patients). Pearson correlation coefficient (r) is shown for each comparison.

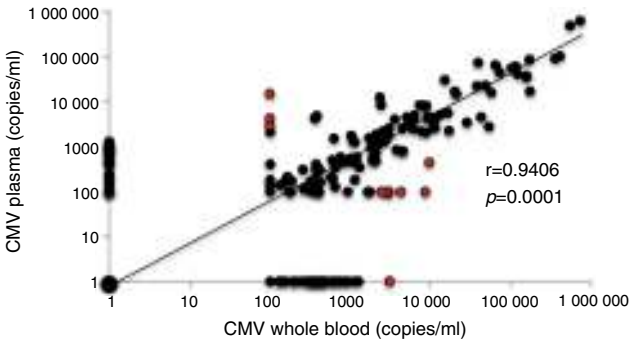

Figure 2 Comparison of the CMV viral load in plasma and whole blood. Pearson correlation coefficient (r) is shown for the comparison. Discordant measurements are shown in red in which the viral load was elevated in plasma, but not in whole blood or vice-versa. A 10-base logarithmic scale was used for the graph in order to better separate the data, although the Pearson correlation was calculated linearly.

in blood ($p = 0.79$). Viral load of CMV in plasma vs. whole blood was compared with the objective of identifying the variation shown between both biological sources (Fig. 2). A positive high and statistically significant correlation was found ($r = 0.9406$, $p = 0.0001$), which indicates that both plasma as well as whole blood reliably reflect the behavior of CMV in the patient with immunosuppressive treatment. In nine measurements, the correlation between samples was very poor, with values > 5000 copies/ml in plasma vs. 100 copies/ml in whole blood or vice-versa, values > 5,000 copies/ml in whole blood and values $\leq$100 copies/ml in plasma. These cases are shown in red points in Figure 2.

With the aim of determining if plasma or whole blood better reflects viral replication, the correlation between viral load in plasma or whole blood and the levels of immunosuppressant were analyzed. According to Figure 3 and as expected, there were very similar degrees of correlation found between the levels of immunosuppressant and viral load, both in plasma as in whole blood. However, this relationship had values very close to zero. The latter indicates that the evaluation of the immunosuppressant in blood does not allow predicting if the patient has an elevated CMV load and therefore is at risk to develop an associated morbidity. For this reason, it is necessary to monitor the viral load together with immunosuppressant levels in the blood. All r values and the respective p value are shown in the graphs.

4. Discussion

As a strategy to prevent graft rejection and graft vs. host disease, patients who receive SOT should be pharmacologically immunosuppressed. However, as a result of this immunosuppression, post-transplant patients are at risk of developing severe infections.[12,17–19] Of particular importance is infection by the herpesvirus family. More than 50% of the adult population is infected by one or various herpesviruses, although the infection is latent and asymptomatic in healthy individuals. The same does not occur in individuals undergoing immunosuppressive treatment. In these patients, the equilibrium of the infection is altered and patients can develop severe, life-threatening diseases. Among the common problems due to herpesviruses that present in patients with immunosuppressive management are severe CMV infections and lymphoproliferative disease due to the Epstein-Barr virus (EBV).[20] On the other hand, reducing the dose of the immunosuppressant drug increases the risk of transplant rejection and graft vs. host disease, which is also associated with increased mortality of the transplant patient.

Despite the increasingly larger number of immunosuppressant drugs available on the market, the risk of severe infection by herpesviruses continues. Tacrolimus and sirolimus are two of the main immunosuppressant drugs used worldwide.[21] Both have very similar structures and recognize FKBP12, a chaperone protein member of the family of the immunophilins.[22] These compounds were originally isolated from *Streptomyces hygroscopicus* by its potent immunosuppressive activity. Because both drugs efficiently and specifically abate the cytotoxic immune response, they are widely used to prevent risk of transplant rejection. However, decrease of the cytotoxic immune response results in loss of infection control by herpesviruses.

This present study analyzed 55 transplant patients treated with sirolimus or tacrolimus from which 541 determinations of the circulating levels of the drugs and of the CMV viral load were carried out. Data obtained show interesting trends and assist clinically in patient follow-up. No significant association was found between medication dose and blood levels. This was reproduced for both

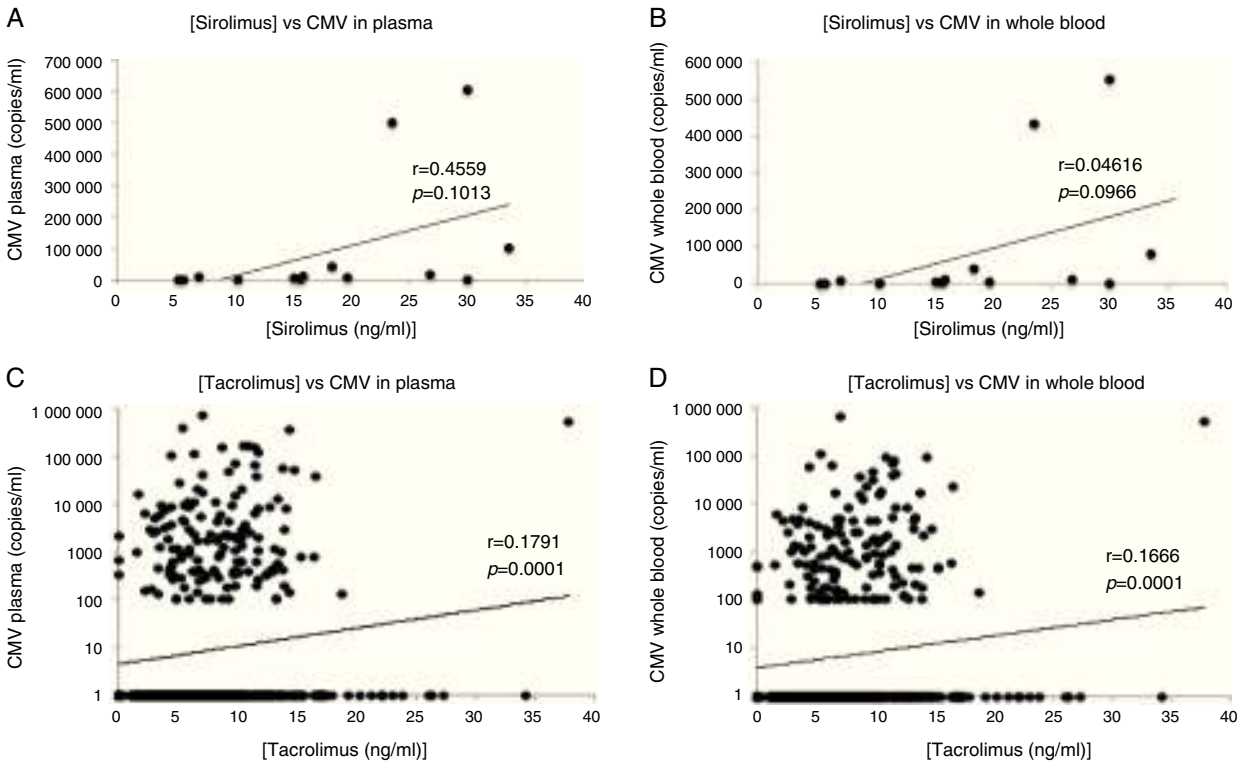

Figure 3 Concentration of sirolimus (A-B) and tacrolimus (C-D) vs. viral load in plasma (A,C) or whole blood (B,D). Pearson correlation coefficient (r) is shown for each comparison. Number of copies of CMV was graphed in a 10-base logarithmic scale in order to better separate the data, although Pearson correlation was calculated linearly.

immunosuppressant agents analyzed indicating that continuous drug monitoring is necessary to have a better idea of its pharmacological effect and patient protection. Both immunosuppressant agents, sirolimus and tacrolimus, have a low therapeutic index. Therefore they are considered as monitoring drugs because their circulation levels must be constantly evaluated.[23] This is due to the variability of the pharmacodynamic and pharmacokinetic characteristics between patients. The data presented agree with the above information.

The herpesviruses have a two-phase life cycle: latent and lytic. The latent phase is associated with a cellular dependency in which there is not production of viral particles. On the contrary, the lytic phase could be associated with viremia. For this reason, it is important to ask oneself: which is the best source of clinical sample for CMV analysis? The data observed support that, for CMV, there is a very close correlation between viral load present in whole blood and in plasma, at least in patients who are pharmacologically immunosuppressed. Therefore, with few exceptions, both plasma as well as whole blood allow us to have a good idea of the viral expansion processes due to *de novo* infection or reactivation that a transplant patient is experiencing. Of the 541 measurements done, in only nine there were values found that did not correlate in which one of the measurements (in plasma or whole blood) showed values $\geq$5000 copies of CMV/ml, whereas the other measurement showed negative or barely detectable CMV values. To date there is no consensus about the levels of CMV that require therapeutic intervention, but the internal recommendation in different transplant centers is from $\geq$5,000 copies of CMV/ml.[24]

These data also support that the continuous monitoring of the viral load in transplant patients is necessary because systemic levels of the immunosuppressant do not permit predicting the behavior of CMV. This is illustrated not only by the values of the Pearson correlation obtained but also by the patients with acceptable immunosuppressive levels or higher than recommended for age (>10 ng/ml) and viral loads > 5,000 copies/ml.

In summary, the dose of the immunosuppressant drug does not correlate with levels in peripheral blood. For this reason, these levels should be constantly monitored along with the CMV load. Viral load measured in whole blood or plasma was found to have a high correlation, indicating that both types of clinical samples are equally reliable in reflecting the processes of infection/reactivation by CMV due to the immunosuppression that the transplanted patient is experiencing. Follow-up of the transplanted patient through quantification of the systemic levels of the immunosuppressant drugs and of the viral loads of CMV allows the physician to intervene before the patient presents a serious clinical complication. This routine analysis of transplanted patients allows for a higher survival rate of the patients and the grafts.

Ethical disclosure

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance

with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Conflict of interest

The authors declare no conflict of interest of any nature.

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