

ANTIBODY AND CYTOKINE SERUM LEVELS IN PATIENTS SUBJECTED TO ANTI-RABIES PROPHYLAXIS WITH SERUM-VACCINATION

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ABSTRACT: Rabies is considered a fatal disease once clinical symptoms have developed. The aim of this study was to evaluate epidemiological aspects and immune response in patients attacked by domestic and wild animals and subjected to post-exposure rabies treatment with equine serum and associated vaccine. Thirty-three patients were evaluated; they were between 13 and 65 years old, 75.8% were male and 24.2% female, and from the Botucatu neighborhood. Twenty healthy control individuals with the same age range were also studied. Specific antibodies to equine immunoglobulins and IFN- γ , IL-2, IL-4, and IL-10 production were evaluated by ELISA. IgM, IgE, IgG and subclasses, and rabies virus antibodies serum levels were determined by nephelometry and seroneutralization methods, respectively. No anaphylactic or serum sickness allergic reactions were observed in patients after treatment. Anti-equine IgG levels were significantly higher than those of IgM after 14 and 28 days of treatment. Protective antibodies to rabies virus > 0.5 UI/ml were detected in 84.6% and 75% of patients at days 14 and 28, respectively. IFN- γ , IL-2 and IL-10 levels in patients before and 48h after treatment were significantly higher than in controls suggesting that both Th1 and Th2 cells were activated in the patients. Serum IgM levels were higher at day 14, and IgG₂ and IgE levels were higher at day 28 of treatment. These results suggest that post-exposure rabies treatment in humans induces significant alterations in patient immune response characterized by increased levels of cytokines, serum levels of specific rabies virus antibodies, and the equine serum components employed in the treatment.

KEY WORDS: rabies, anti-rabies prophylaxis, immunoglobulins, cytokines.

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INTRODUCTION

Rabies is a zoonotic, viral disease. The causative agent, *Lyssavirus* of the family *Rhabdoviridae*, is responsible for acute encephalitis. In developed countries, rabies is spread from wild to domestic animals and consequently to humans; once clinical signs appear, it is fatal. (10).

From an epidemiological point of view, rabies remains a very important public health problem in several developing countries from the African, Asiatic (64), and South American continents (15, 28, 43, 45, 46, 63, 64). It is estimated that around 300 people die from the disease and approximately 300,000 are treated every year with post exposure serum vaccination (2).

Oceania and Antarctica are free of the disease (2, 37, 50), and it has been eradicated in Japan, England, the Oceania islands, some Pacific islands, and other island countries. In countries such as France, Germany, Spain, Canada, and the United States, it has been controlled by efficient surveillance systems (39, 44).

In Brazil, despite great advances in rabies control, there are still incidences of the disease in the North, Northeast, Central West, and some southern states (47). The southern region is considered a controlled area (45). Reports of the disease have been decreasing in Brazil since the 1980's, but case frequency is still variable. In 2000, twenty-six cases were recorded and confirmed, four of which were in the Central West, nine in the North, and 13 in the Northeast (15). The highest incidences occur in poorer areas, and in areas where there are no effective control programs and there is higher contact between man and animals.

The World Health Organization (WHO) recommends that all people highly exposed to animals with suspected or confirmed rabies diagnosis must receive anti-rabies prophylactic treatment with serum vaccination (60). This treatment is intended to protect the host against disease development using the following strategy: the anti-rabies serum specific immunoglobulin provides a long period of protection, offering antibodies for immediate virus neutralization, while the vaccine stimulates endogenous antibody production to give an efficient immunologic memory (14, 61).

Equine anti-rabies serum is a purified immunoglobulin solution with antigenic properties which induce IgE antibodies formation by a sensitization process and may cause anaphylaxis on subsequent serum exposure and form immune complexes which can trigger serum sickness (5, 9, 12, 25, 27, 38, 54). Kaliner & Belevel (26) reported that equine anti-rabies serum caused reactions in 64% of cases.

Heterologous proteins trigger a complex immunological process in the receptor organism which in turn triggers hypersensitivity mechanisms (51).

Pre and post-exposure prophylaxis regimens are well established today by the simultaneous use of vaccine and hyperimmune serum. Serotherapy should be used with caution because it may trigger major reactions in the receptor, although with relatively low frequency and severity. Administration is therefore recommended in hospitals prepared to deal with adverse reactions.

Since both heterologous anti-rabies serum and rabies vaccine have immunogenic determinants, they can induce cellular and humoral immune response in patients subjected to serum vaccination treatment. This study evaluated the specific antibodies produced against equine immunoglobulin and rabies virus, and the serum immunoglobulin and cytokine levels in patients subjected to post-exposure rabies prophylactic treatment after domestic animal aggression.

PATIENTS AND METHODS

Patients

There were 33 patients, 25 males and 8 females, with ages ranging from 13 to 65 years old (mean: 40.6 years old); they were admitted to the Tropical Diseases Clinic at the University Hospital of the Botucatu School of Medicine, São Paulo State University, UNESP, between January 2000 and October 2001. They reported exposure to animals with suspected rabies: 12 had been exposed to biological material from bovine with suspected rabies; three had contact with wild animals, two with simian and one with a bat; and 15 had been victims of aggression from domestic animals, canine (seven), feline (five), and swine (three). A group of 20 healthy blood donors, gender and age matched to patients, was included as controls. This study was approved by the Ethics Committee of the Botucatu School of Medicine, and informed consent was obtained from all patients, controls, or their parents.

Treatment

Patients exposed or attacked by rabies-suspected animals were considered at risk of contracting the rabies virus. Thus, as per World Health Organization recommendations they received anti-rabies prophylactic treatment with serum-

vaccination (45) consisting of passive immunization with 40 UI/kg equine heterologous serum by intramuscular (IM) route. Active immunization was administered with human anti-rabies vaccine prepared from the rabies virus strain WISTAR PM/WI 38-1503-3m, cultured in Vero cells (Pasteur Mérieux); it was also administered by IM route in a five-dose schedule: the first dose on the day of virus exposure and the others at days 3, 7, 14, 21, and 28 after exposure. The patients were questioned about the time elapsed between the accident and medical attention, and about any previous administration of anti-rabies serum.

Human sera

Blood samples (6 ml) were collected without anticoagulant in 10-ml plastic tubes before serum vaccination treatment and then after 48 h, 14 and 28 days of treatment. Blood was centrifuged at 2500 rpm for 10 min; the serum was allowed to separate and was then stored at -20°C until determination of specific antibodies to virus, equine immunoglobulin, human serum immunoglobulins and cytokines.

Anti-equine IgG and IgM immunoglobulin determination

Serum levels of anti-equine immunoglobulin antibodies in patients subjected to serum-vaccination treatment were determined by enzyme-immunoassay (ELISA). Linbro Titertek (Flow Laboratories, Inc., Mclean, VA, USA) 96-well plates were coated overnight at 4°C with 100 µl of 1:8000 dilution F(ab)2 fraction of equine anti-rabies in 0.05 M carbonate buffer, pH 9.6. The plates were then washed with phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (T-PBS). At this and all other steps, wells were washed six times with 200 µl T-PBS. The blocking solution containing 2% bovine albumin (Sigma Chemical Co., St Louis, MO, USA) and 0.05% gelatin (Sigma) in 200 µl PBS was added and plates were incubated for 60 min at 37°C and washed six times with T-PBS. Sera of patients or controls (100 µl) were added in duplicate to the wells and incubated for 60 minutes at 37°C. After washing, 100 µl of 1:1000 rabbit IgG, anti-human IgG, or 1:2000 anti-human IgM conjugated with peroxidase (Sigma Chemical Co., USA) was added to each well, and the plates were incubated for 60 min at 37°C. Wells were then washed with T-PBS and 100 µl of an enzymatic substrate containing 0.4 mg/ml and 2 µl/ml of hydrogen peroxide in 0.1 M citrate phosphate buffer, pH 5.2, was added. After incubating at

room temperature in the dark for 15 min, the reaction was stopped by adding 50 µl of 2 M sulphuric acid, and absorbance values were measured at 492 nm using an ELISA reader (Multiskan Spectrophotometer, EFLAB, Helsinki, Finland). Results were expressed as optical density (OD). Reaction cutoff was established as the highest serum OD value obtained from 13 healthy individuals tested at 1:200 dilutions. The OD of each patient serum dilution was compared with reaction cutoff. OD values above 0.141 for IgG and above 0.183 for IgM were considered as positive.

Anti-rabies virus antibodies determination

Neutralizing antibodies were measured by Simplified Fluorescent Inhibition Microtest [SFIMT] (20). Briefly, the test sera and positive and negative standard sera were distributed into a series of two-fold dilutions in minimum Eagle medium (MEM) containing 10% bovine fetal serum beginning from 1/5 in 100 µl per well of Linbro Titertek (Flow Laboratories) 96-well plates; 50 µl of a previously titrated optimal viral suspension (PV virus) was added to each well to infect 80% of the cells; this was followed by an incubation period of 60 min at 37°C. Then, 50 µl BHK₂₁ cells (clone 13), growing in MEM with 10% bovine fetal serum (10⁶ cells/ml), was added to each well. The microplates were incubated at 37°C for 24 h in a humidified CO₂ atmosphere. Virus infection rate was determined in each test. After cell fixation with 80% cold acetone for 15 min, cells were stained by an anti-rabies nucleocapsid fluorescent conjugate (Sanofi cod 72114). The plates were washed with PBS followed by distilled water. Then, 50 µl of 0.5 M glycerine buffered carbonate, pH 9.6, was added to each well. The plates were examined in an epi-fluorescence inverted microscope to evaluate the effect of the virus on the cell culture. Results expressed the dilution corresponding to the well with 50% decrease of infection. Comparison of test sera results with those of standard serum were used to obtain titer IU values.

Immunoglobulin determination

The IgM, IgG and isotypes, and IgG1, IgG2, IgG3 and IgG4 levels were determined in serum of patients subjected to serum vaccination therapy evaluated before and after 14 and 28 days of treatment. IgM and IgG were determined by nephelometry using binding site antibodies, reagent Kit (Behring, Birmingham, UK), according to

the manufacturer's instructions. Immunoglobulin concentrations were measured with a BN 100 nephelometer (Dade Behring, Marburg, Germany) and ARRAY 360 equipment (Beckman Coulter, Inc., Los Angeles, CA, USA). Immunoglobulin isotype results were expressed in mg/100ml.

IgE determination

IgE serum levels were evaluated by an Elecsys Enzymun-test (Roche, diagnostic Corp., Indianapolis, IN, USA) with a ruthenium-labeled anti-IgE monoclonal antibody. Reactions were developed according to the manufacturer's instructions, and IgE concentrations were obtained in an ELECSYS 2010 apparatus (Roche, Diagnostic Corp., Indianapolis, IN, USA).

Enzyme-linked immunosorbent assay for cytokines

Cytokines IL-2, IL-4, IL-10, and IFN- γ concentrations were determined in patient serum obtained before and after treatment (48 h later) by ELISA. Antibody matched pairs and respective standards were purchased from R&D Systems (Minneapolis, MN, USA) and used according to the manufacturer's instructions. Assay sensitivity limit was 10 pg/ml for all cytokines tested.

Statistical analysis

Data were statistically analyzed using InStat software (Graph Pad, Software, version 3.05, San Diego, CA, 2000). Serum levels of anti-equine immunoglobulin antibodies and anti-rabies virus found in patients before and after treatment and in the control group were compared by one-way analysis of variance (ANOVA), followed by multiple comparisons by the Tukey-Kramer method, and concentrations of IgE, IgM, and IgG isotypes were analyzed by the paired t-test. Differences in the cytokine responses between patients and controls were determined by Kruskal-Wallis non-parametric analysis of variance, followed by Dunn Test multiple comparison. Significance level was set at $p < 0.05$ in all analyses.

RESULTS

We studied the epidemiological characterization of 33 patients receiving rabies serum vaccination after exposure to domestic or wild animals. The patients showed

high exposure to bovine biological material followed by canine and feline attack, animals of major epidemiological importance.

Bite was the major route (54.6%), followed by animal manipulation (42.4%), allowing contact with biological materials (blood and saliva), and bite followed by scratching (3%). Absence of wound was seen in 42.4%, multiple lesions in 33.3%, and single lesion in 24.2% of cases. Lesions were mainly in the upper limbs (81.2%), inferior limbs (9.4%), and both (6.2%); there were only 3.1% in the trunk.

Post-exposure serum vaccination was indicated for all patients in the study, using a five-dose regimen of Vero cell culture produced vaccine in combination with the equine anti-rabies serum containing 40 UI/kg purified equine immunoglobulin as per the Pasteur Institute Manual (45). No adverse reactions were seen in either vaccine or anti-rabies serum inoculation sites. Also, patients did not develop either anaphylactic or serum sickness hypersensitivity reactions.

Anti-equine immunoglobulins antibodies were detected in the serum samples of patients subjected to serum-vaccination treatment and in the control group. Figure 1 shows that patients developed anti-equine IgG and IgM immunoglobulin antibodies after treatment. Both IgM and IgG antibody levels detected at 14 and 28 days of treatment were significantly higher than before treatment or in healthy controls. IgG serum levels were significantly higher than IgM at both 14 and 28 days after treatment. At day 28, IgG levels were significantly higher than at day 14, while no significant differences in IgM levels were observed in these periods.

The analysis of neutralizing anti-rabies virus antibodies in patient sera before and after vaccination showed that patients did not have antibody protection before vaccine administration. After treatment, patients had clearly developed high levels of specific antibodies against rabies virus at both 14 and 28 days of vaccine regimen. Antibody levels in patient serum at these times were not significantly different. Based on WHO criteria (62) proposing a 0.5-UI/ml limit for establishing serum conversion after rabies vaccination, 11/13 patients (84.6%) at 14 days and 15/20 patients (75%) at 28 days treatment were positive for serum conversion.

Figures 2 and 3 show serum concentrations for total IgM and IgG and IgG1, IgG2, IgG3 and IgG4 subclasses in patients before and after 14 and 28 days of treatment regimen, respectively. Figure 4 shows IgE levels. Immunoglobulin levels after treatment were generally higher than before treatment. Patients presented

significantly higher levels of IgM at 14 days treatment (Figure 2) and IgG2 and IgE at 28 days treatment (Figures 3 and 4).

Table 1 shows IFN- γ , IL-2, IL-4, and IL-10 in sera of both equine-immunoglobulin and rabies-vaccine treated and untreated patients compared to levels in healthy controls not subjected to serum vaccination treatment. IFN- γ , IL-2, and IL-10 levels were significantly higher in patient groups both before and after treatment compared to those in the control group. No significant difference was observed between groups for IL-4 levels.

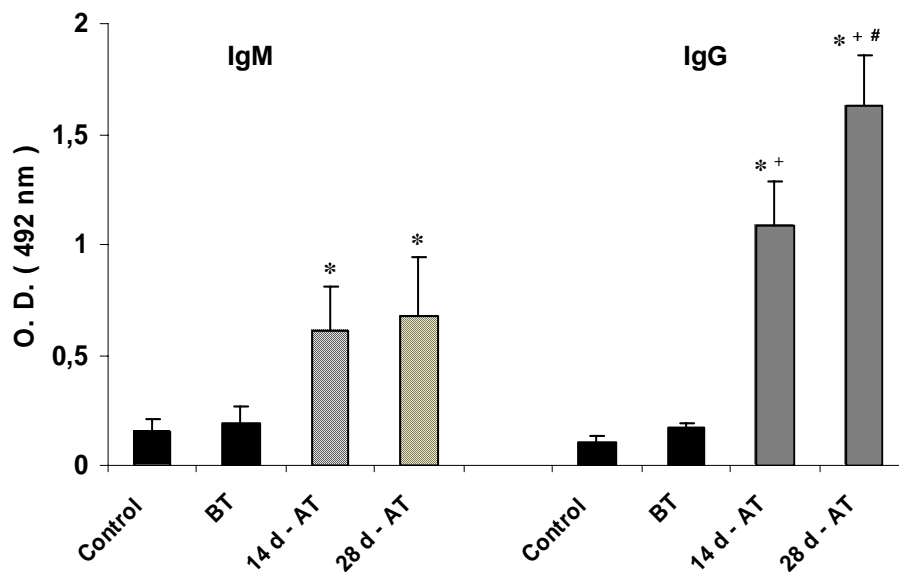


Figure 1: Anti-equine IgM and IgG immunoglobulin antibodies in sera of patients evaluated before (BT) and after (AT) treatment with rabies serum vaccination. Results are expressed as mean \pm SD of optical density (OD)

* ($p < 0.05$) versus control, BT;

+ ($p < 0.05$) versus IgM;

($p < 0.05$) versus 14d-AT (ANOVA).

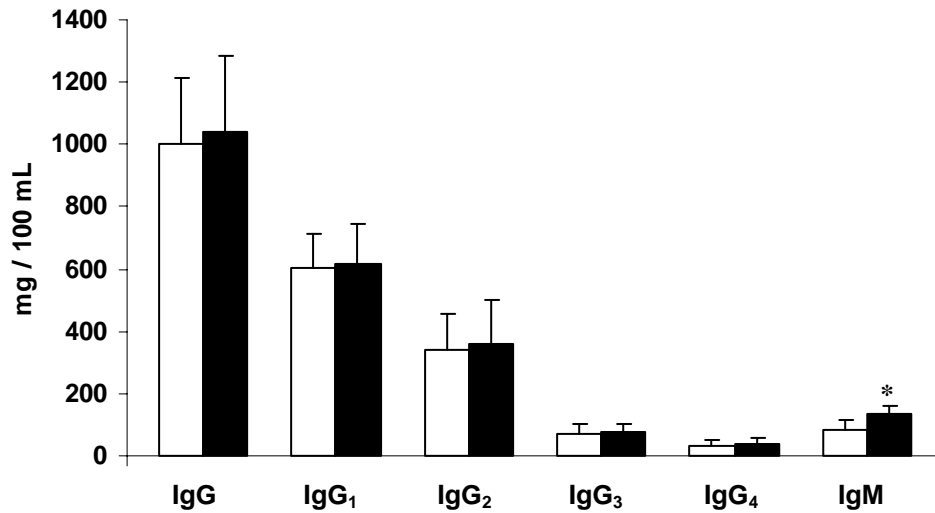


Figure 2: IgG and subclass serum levels in patients evaluated before and after 14 days of treatment with rabies serum vaccination. Results are expressed as mean \pm SD of mg/100 ml.

*($p < 0.05$) paired t test

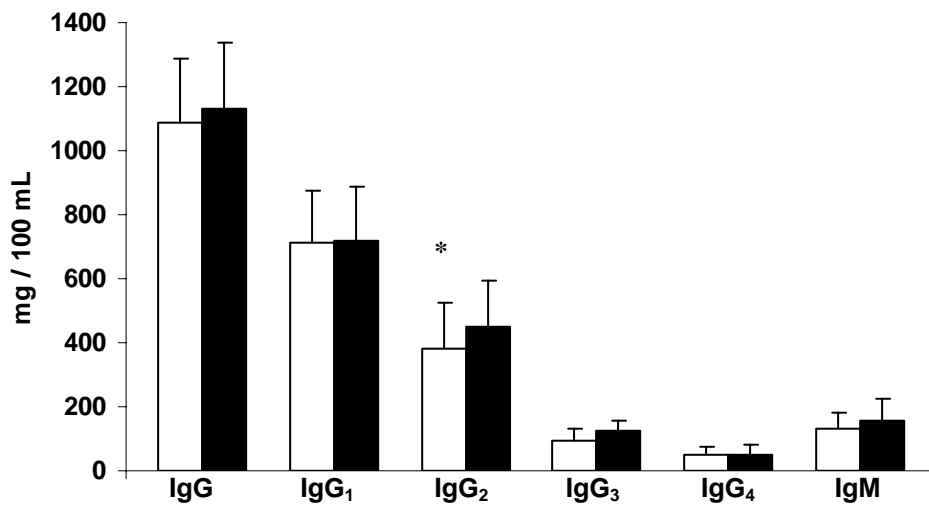


Figure 3: IgG and subclass serum levels in patients evaluated before and after 28 days of treatment with rabies serum vaccination. Results are expressed as mean \pm SD of mg/100 ml.

*($p < 0.05$) paired t test

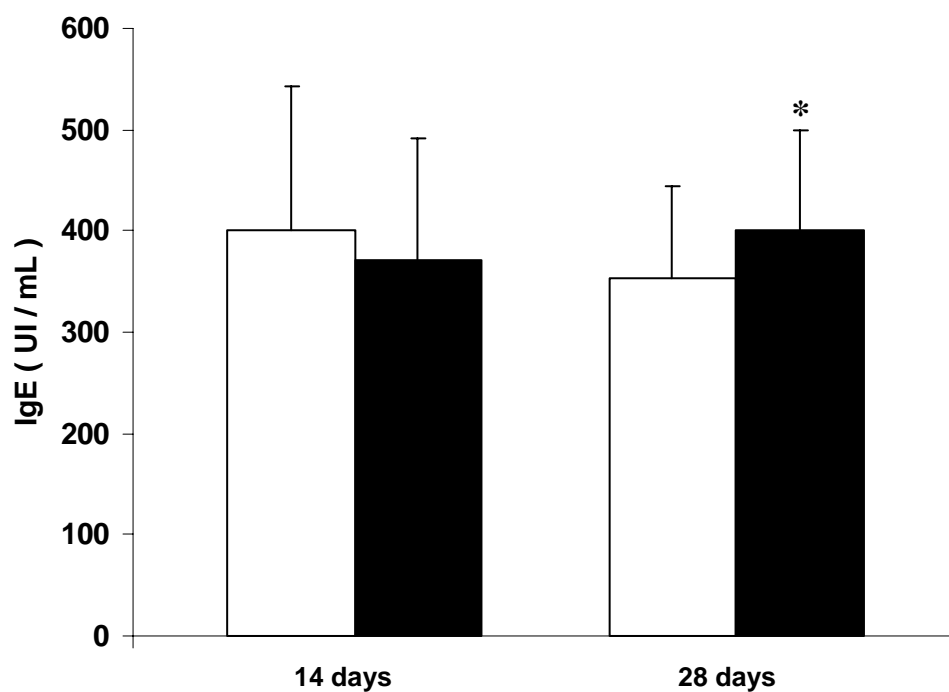


Figure 4: IgE serum levels in patients evaluated before and after 14 and 28 days of treatment with rabies serum vaccination. Results are expressed as mean \pm SD of UI/ml.

* ($p < 0.05$) paired t test

Table 1: Serum levels of Interferon-gamma (IFN- γ), Interleukin-2 (IL-2), IL-4 and IL-10 detected in 33 patients evaluated before and after 48 h of treatment with equine immunoglobulin and in 20 healthy controls.

PATIENTS	IFN-γ	IL-2	IL-4	IL-10
Patients evaluated before treatment (G1) (n=33)	346.0 (217.0 – 610.0)	134.0 (97.0 – 321.0)	5.0 (0 – 15.0)	15.0 (0 – 31.0)
Patients evaluated after treatment (G2) (n=33)	358. (217.0 - 717.0)	148.0 (94.0 – 368.0)	6.0 (0 – 15.0)	16.0 (0 – 36.0)
Controls (G3) (n=20)	213.0 (98.0 – 310.0)	107.5 (63.0 – 183.0)	8.0 (0 – 15.0)	2,5 (0 – 16.0)
Significance*	G1 = G2 > G3 ($p < 0.01$)	G1 > G3 ($p < 0.05$) G2 > G3 ($p < 0.01$)	NS	G1 > G3 ($p < 0.05$) G2 > G3 ($p < 0.01$)

Results are expressed as median values and range (in parentheses).

* *Kruskal-Wallis test*

DISCUSSION

Human rabies prevention using serum and vaccine is undoubtedly the only prevention method available today (4, 21, 23, 30) and has satisfactorily contributed to decreasing the number of rabies cases in Brazil since the 1980's (44, 48).

Patients evaluated at both 14 and 28 days after anti-rabies serum vaccination treatment showed significantly higher anti-equine IgM and IgG immunoglobulin levels than pre-treatment or healthy individuals. These results show the body's capacity to produce antibodies against equine antigens persistent in the patients' circulation, suggesting that equine protein is highly immunogenic to man. Also, IgG levels were

significantly higher than IgM in both studied groups. Although there was no significant difference between IgG results at days 14 and 28 after treatment, there was a tendency to higher values in patients evaluated at day 28. The fact that equine antibody half-life is approximately 21 days in serum and that they can be detected up to 44 days after treatment corroborates this hypothesis (7,8,13, 55, 58).

Although patients in this study produced IgM and IgG antibodies specific to anti-rabies serum proteins, they did not develop clinical signs compatible with anaphylactic reactions or serum sickness. Treatment therefore characterized the sensitization process but not the formation of sufficient immune complexes to cause serum sickness. This suggests that equine immunoglobulin stimulates the immune system into forming specific antibodies, but without causing adverse effects; this is probably due to the serum purification process during production. There has been a low incidence or absence of adverse reactions in patients treated with purified equine immunoglobulin after exposure to rabies (31). In the past, the use of non-purified equine heterologous serum induced serum sickness in approximately 15% of children and 40% of adults (26). Pre-exposure to equine proteins found in animal hair, serum, and skin peeling products can induce prior sensitization making patients prone to serum sickness after treatment with immunobiological agents (3, 9, 35). Kaliner & Beleva (26) reported that adverse reactions in patients treated with heterologous serum depend on their prior exposure to equine immune serum, produced and purified by different methods, and volume of serum administered. Equine heterologous serum purification makes it more efficient and safe in treating patients who require serotherapy (64, 65).

We evaluated anti-rabies immunization efficiency during a vaccine regimen at days 14 and 28. We detected seroconversion in 84.6% of patients at day 14 and 75% at day 28 without significant difference between groups for neutralizing antibody levels. Literature reports that the production of neutralizing antibodies in protective titers (>0.5 UI/ml) occurs in all patients subjected to serum vaccination from the 14th day of active immunization (16, 24, 31, 52, 58). Therefore, we can consider that the serum vaccination regimen used in this study did not attain the desired protection level for all patients throughout the study period. The lack of serum conversion could be associated with simultaneous administration of the vaccine and anti-rabies serum. Non-adherence of some patients to active immunization after hospital treatment could be considered a failure factor in anti-rabies protection (65, 66).

IgM, IgE, and IgG immunoglobulin class and subclass values in patients treated with anti-rabies serum vaccination were higher than pre-treatment values. Patient IgM levels were significantly higher at day 14, and IgG2 and IgE were higher at day 28 than in pre-treatment, demonstrating antigenic stimulation which could be associated with the response to equine immunoglobulin antigens and viral antigens. Therefore, equine immunoglobulin serotherapies for post-exposure rabies treatment and transplant rejection have shown IgM and IgG antibody production specific to the equine protein present in the immunobiological agent (11, 22, 35, 36, 59).

A comparative study on the production of IgG subclasses specific to different vaccination regimens using vaccines prepared in human diploid cells and obtained in nerve tissue, type Fuenzalida & Palacios, showed that the IgG1 subclass was the main immunoglobulin produced regardless of vaccine regimen and vaccine used. However, individuals vaccinated by intramuscular route had higher IgG2 positivity than those vaccinated via intradermal route (18).

The significantly higher IgG levels at 28 days of treatment with serovaccination could have been due to immunization with vaccine prepared in cell cultures. Some authors have reported IgE specificity in a small number of immunized individuals with vaccines prepared in cell cultures and directed against beta-propiolactone and human albumin, components used in the preparation of vaccines which are responsible for inducing allergic reactions in vaccinated individuals (18, 42, 53).

Therefore, results suggest that both the immunological agent and the vaccine used to treat patients show many immunogenic epitopes capable of inducing specific immune responses detectable by ELISA and seroneutralization. Polyclonal activation induced by these antigens seems to have been sufficient to increase total post-treatment immunoglobulin levels.

Analysis of serum cytokine production in patients subjected to anti-rabies serum vaccination showed that IFN- γ , IL-2, and IL-10 levels were significantly increased before treatment. This can be attributed to the physical and psychological stress to which the patient was subjected, starting with the attack and lasting until medical assistance, which varied from 4 to 18 hours. Literature shows that human psychological stress can suppress or activate immune response functions and interfere with cytokine synthesis (6, 29, 32, 56). Significantly elevated levels of TNF- α , IL-6, IFN- γ , and IL-10 were seen in individuals subjected to psychological stress (32). High psychological stress situations shift the cytokine balance from Th1 to Th2,

resulting in decreased IFN- γ production and increased IL-10 synthesis (33), while stress-induced anxiety state is related to Th1 pattern response with increased IFN- γ and less IL-4 and IL-10 production. These results indicate that changes in the production of pro-inflammatory cytokines, TNF- α , IL-6 and IFN- γ , and IL-4 and IL-10, considered negative immunoregulatory cytokines, play a major role in homeostasis regulation due to psychological stress (32).

Cytokine levels after 48 h of serovaccination treatment showed persistently high IFN- γ , IL-2, and IL-10 values. These suggest that, in addition to stress effects, immune system stimulation by virus-exogenous antigens and administered equine immunoglobulin could be responsible for the sustained production of these cytokines. Previous studies have shown that anti-rabies vaccination induces IFN- γ production (46) in mice with correlation between cytokine production and degree of resistance to the virus (19). T CD4⁺ lymphocyte clones specific for G & N proteins of viral nucleocapsid, producers of IFN- γ and IL-2, have been detected in individuals immunized against rabies (17, 67). Also, lymphocytes in vaccinated individuals specifically respond to antigens from different virus samples with IL-2 production, suggesting that this cytokine can be considered a good parameter for studying cell immunity and T memory cell induction by anti-rabies vaccination (41).

Evidence from literature on Th1, IFN- γ , and IL-2 cytokine production by T cells from vaccinated individuals supports our hypothesis that high levels of these cytokines in patient sera may result from stimulation by the vaccine virus. Anti-virus neutralizing antibody production, equine anti-globulin IgM and IgG synthesis, and higher IgG2 serum levels in patients evaluated at day 28 suggest IFN- γ and IL-2 participation in inducing the immune response to serum vaccination. Therefore, IFN- γ induces protective antibody response which participates in phagocytosis and elimination of microorganisms by macrophages, which are also activated by this cytokine, while IL-2 acts predominantly on cell immune response stimulation (1).

However, maintenance of high IL-10 levels in patients evaluated after treatment suggests a cytokine modulatory role on the immune response. IL-10 is mainly produced by human Th2 cells (57), macrophages, and B cells (40). This cytokine stimulates B lymphocyte proliferation aiding humoral response while it has an inhibitory effect on IFN- γ production (1). Elevated IL-10 levels can be produced by peripheral blood human lymphocytes stimulated by rabies viral nucleocapsid,

considered a superantigen capable of inducing B and T cell polyclonal activation (49). The fact that viral superantigen preferentially activates Th2 cells (34) seems to explain the increased production of antibodies in serovaccination-treated patients in our study.

As high IL-10 levels have been detected 48 h after serum vaccination treatment, their production may be mainly related to neuroendocrine circuit homeostasis maintenance, caused by psychological and physical stress in patients after attack, and B cell polyclonal activation induced by the superantigen action of virus components, then involved in immune response regulation triggered by serum vaccination treatment.

Together, the results of this study show that anti-rabies serum vaccination induces significant alterations in the immune response of treated patients, demonstrated by the production of cytokines and anti-virus specific antibodies and equine anti-immunoglobulin.

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