Skin Window Immune Response to Normal Human IgG in Patients with Rheumatoid Arthritis and Acute Poststreptococcal Glomerulonephritis

BERNARDO RODRIGUEZ-ITURBE, M.D., VICTOR SILVA-BEAUPERTHUY, M.D., GUSTAVO PARRA, M.D., LIRIMO RUBIO, M.D., AND ERNESTO GARCIA, M.D.

Rodriguez-Iturbe, Bernardo, Silva-Beauperthuy, Victor, Parra, Gustavo, Rubio, Lirmo, and Garcia, Ernesto: Skin window immune response to normal human IgG in patients with rheumatoid arthritis and acute poststreptococcal glomerulonephritis. Am J Clin Pathol 76: 270-275, 1981. The skin window technic was utilized to determine the reactivity of patients with rheumatoid arthritis (RA) and acute poststreptococcal glomerulonephritis (APSGN) to human IgG (H-IgG). The response to H-IgG was compared in nine patients with RA, 20 patients with APSGN, and 10 normal individuals. All subjects were tested concomitantly with the saline solution used as solvent for H-IgG. The normal controls and five patients were challenged, in addition, with diphtheria-tetanus-pertussis antigen (DPT) to which they had previous prophylactic exposure.

The following results were obtained: 1) Four patients with RA and nine patients with APSGN responded with increased lymphocyte migration (more than 2 SD above the normal mean level) at nine and 12 hours. 2) The mean estimated immunogenic lymphocytosis (calculated subtracting the lymphocyte counts of the saline skin windows) of both patient groups was significantly higher than that of controls at the same time intervals. 3) The response of normal individuals and patients to DPT was comparable in time of appearance and intensity to the response of patients to H-IgG.

Our studies suggest that patients with RA and APSGN respond to H-IgG in a manner comparable to that observed with a known antigenic stimulus and support a clinical role for antiglobulin reactivity. (Key words: Skin-window; Antiglobulins; Rheumatoid arthritis; Glomerulonephritis.)

Materials and Methods

The following groups of individuals are the subject of this study: a) Patients with acute poststreptococcal glomerulonephritis. Twenty patients with well established diagnosis are in this group. Each patient was admitted to the Hospital Universitario with acute nephritic syndrome and each had evidence of preceding streptococcal infection as indicated by elevated antistreptococcal antibody activity (antistreptolysin O, antihyaluronidase or streptozyme test). The site of antecedent infection was the throat in eight patients and the skin in ten patients. The site of infection was unknown in two patients. There was no evidence of active skin infection in any of the patients at the time of the skin window testing, which was performed prior to discharge from the hospital, an average of four weeks after admission (range: 20 days to 37 days). The patients were equally divided by sex and their age ranged from eight to 18 years. They were receiving no medication or alternatively, whether the antiglobulin phenomenon has little clinical significance.

In order to examine this question the skin window technic was used to study the response to human IgG in patients with acute poststreptococcal glomerulonephritis and rheumatoid arthritis. The investigation was performed with concomitant testing of each individual with the saline solution used as solvent for IgG. The results in our patients indicate a lymphocyte migration with IgG which was comparable in intensity and time of appearance to that observed with diphtheria-tetanus-pertussis antigen. Since the response to IgG was not found in normal individuals, the findings suggest an immunogenic role for IgG in these disease states.
at the time of the study. b) Patients with rheumatoid arthritis. There were nine patients in this group. Seven of them were females. All these patients had classical rheumatoid arthritis and were selected from the Arthritis Clinic of the Hospital Central (Maracaibo). The diagnosis of rheumatoid arthritis had been made two–five years previously and the onset of their disease was estimated to range from two to 11 years prior to the study. Their ages varied from 42 to 64 years. None of these patients were on steroid medication but used salicylate preparations for symptomatic relief. c) Control group. Ten apparently healthy individuals were used as control subjects. They were physicians or laboratory personnel of the Renal Service and Laboratory and they had normal serum C3 levels, negative rheumatoid factor titers, and normal serum creatinine, urine analysis and blood pressure. Their age range was 22 to 41 years.

All the subjects of this study had prior immunization against tetanus. All, except two patients with rheumatoid arthritis, were also vaccinated or suffered diphtheria and whooping cough. Informed consent was obtained from the patients and/or their parents as well as from the control individuals.

Skin Window Technic and Reagents

The skin window was prepared by scarification of an area of about 3–4 mm in diameter in each forearm using a scapel tip as detailed by Rebuck and co-workers. The areas were swabbed with 70% alcohol and allowed to dry prior to scarification. Using a graduated insulin syringe, 0.05 ml of the appropriate reagent was dropped on the skin window which was then covered with a sterile round glass coverslip, that in turn was tapped to the forearm. The coverslip was replaced with sterile precautions, three hours, six hours, nine hours, 12–24 hours, and 24 hours later. Each coverslip was air dried and stained with Wright’s solution, and the cells attached to it were examined with the help of a Leitz Ortholux microscope (Wetzlar, West Germany).

The identification and counting of the cells on the slides was done by the same investigator in every case, without prior knowledge of the individual to whom it belonged, nor the reagent that had been applied, not the time interval it represented. In each coverslip, two representative areas of 1.5 mm diameter (×10 magnification) were selected and a differential count was performed in each area. Whenever possible, at least 500 cells were counted. This counting routine was repeated 1–3 weeks afterwards. By limiting the identification and counting to one experienced observer (hematopathologist), the results were repeatable within ±15%. Since there was only one coverslip for analysis in each time interval, no special stains could be utilized for separation of macrophages and differentiating lymphocytes; however, because of the nature of the study, special care was devoted to lymphocyte identification. Cells were classified as polymorphonuclear leucocytes (PMN), lymphocytes, monocytes, and other cells. The latter included macrophages, eosinophils, basophils, and not readily identifiable cells. There was no local evidence of infection in any of the subjects here reported. However, one normal individual, one patient with poststreptococcal nephritis, and one patient with rheumatoid arthritis did present a local infection after 12 hours which required their disqualification from this investigation.

One skin the windows, the following reagents were applied: a) Normal Human IgG (H-IgG), obtained from apparently healthy donors with negative rheumatoid factor activity and isolated and characterized by methods already described. The individuals of the control group here reported were not used as IgG donors. The isolated IgG was lyophilized and redissolved in 0.9% saline solution, passed through Millipore filters (Millipore Corporation, Bedford, Massachusetts, 0.8, 0.45, and 0.22 um) and adjusted to a final concentration of 20 mg/ml in the saline solution. b) Sterile 0.9% Saline Solution (Laboratorios Biofar, Caracas, Venezuela), c) Diphtheria-Tetanus-Pertussis (DPT) Antigen used in Venezuela for prophylactic vaccination and commercially available (Swiss Institute of Vaccination, Bern, Switzerland). This preparation contains 50 U of purified dipheria antigen, 20 U tetanus antigen, and 40 × 10⁹ Bordetella Pertussis per 1 ml.

The experimental design included skin windows in each forearm in every patient. H-IgG was applied to one forearm and saline solution to the other. In addition, the control subjects and five patients had DPT applied on a third skin window done concomitantly.

In order to assess the reproducibility of a cellular response in a given person, two skin windows were prepared in five individuals (two patients and three controls) and H-IgG was placed on each side. The total and differential cellular counts did not differ in one side with respect to the other for more than 15%.

Calculations

Values expressed in the paper as absolute number of cells, refer to the 1.5 mm area were the counts were made, as discussed before. In some analyses, the lymphocyte count in the saline skin window was subtracted from that obtained with H-IgG or DPT in the opposite forearm in the same patient. The latter calculation is referred to as "estimated immunogenic lymphocytosis."
Standard t statistical analysis was employed for the levels of significance. An individual variation is discussed as elevated or high when exceeded two SD the corresponding mean normal value.

Results

Table 1 shows the mean PMN, lymphocyte and monocyte counts of the skin windows at six hours, nine hours, and at 12–14 hours. The counts at three hours were closely similar for all groups under study and for all reagents tested. At this early time, the mean total cellularity ranged from 1072 to 2072 cells with over 80% PMN leucocytes. The total cellularity of successive hours can be approximated from the table, since the cells which were not PMN, lymphocytes or monocytes (“other cells”, see methods) were never above 5% of the total. It can be appreciated from the table that PMN are the predominant type of cell at six hours and, to a lesser degree, at nine hours. At 12–14 hours the monocytes have increased in number to levels which are close to those of PMN cells. Lymphocytes are seldom found before the sixth hour and increased up to the 12–14th hour of the study.

In the saline skin windows the lymphocyte response makes up a maximum of 3–4% of the total cellular response. The H-IgG skin windows in the control subjects had low lymphocyte counts at 12–14 hours (3.6% ± SEM 0.68) while in the patients with poststreptococcal glomerulonephritis and rheumatoid arthritis, the lymphocytes represented at this time 30.0% (±5.86) to 46.3% (SEM ± 13.18) of the total cellular migration. As shown in Table 1, there was great individual variability in each group of subjects. Only the mean lymphocyte count in the patients with poststreptococcal nephritis at nine hours was statistically different from the corresponding value in the control group. The skin windows with DPT in the control individuals had lymphocyte migration in the order of magnitude of those seen in both patient groups tested with H-IgG (Table 1).

The lymphocyte counts of the individual patients are shown in Fig. 1. It can be appreciated that nine out of 20 patients with poststreptococcal glomerulonephritis had skin window lymphocytosis with H-IgG which exceeded 2 SD the mean value observed in controls with the same reagent. Four out of nine patients with rheumatoid arthritis had high values at 14 hours.

The reactivity to H-IgG was also analyzed in comparison with the response to saline solution carried out at the same time. For this analysis, the lymphocyte count of the saline skin window was subtracted from that of the paired H-IgG skin window. Table 2 demonstrates that when studied in this fashion, the mean
values of both patient groups are significantly higher than the mean value of the control individuals. In addition, the estimated immunogenic lymphocytosis in the controls in response to DPT is comparable to that observed in the patients tested with H-IgG. Fig. 2 shows the individual patients in this analysis. It can be seen that at 12–14 hours, nine patients with poststreptococcal glomerulonephritis and four patients with rheumatoid arthritis had a significant estimated immunogenic lymphocytosis in response to H-IgG.

Attempts were made to correlate the skin window results with rheumatoid factor titers and serum C3 levels, but no relationship could be established. Rheumatoid factor titers in patients with rheumatoid arthritis ranged from 1:80 to 1:320, and in the patients with nephritis from negative to 1:160. Serum C3 levels were within normal limits (140 mg/dl ± 22 15) in eight patients with rheumatoid arthritis and elevated in one patient (240 mg/dl). Fifteen patients with poststreptococcal glomerulonephritis had low C3 levels, and five had values in the normal range at the time of skin window testing.

Discussion

Circulating antiglobulins are a feature of the disease under investigation. Serum antibodies to IgG are a highly characteristic of rheumatoid arthritis.7,8 Acute poststreptococcal glomerulonephritis has been reported to be associated with high rheumatoid factor titers13 and some investigators suggest that antiglobulin formation and deposition could be a central pathogenetic event in the disease.12 However, the commercial preparations used for the determination of rheumatoid factor differ in sensitivity, and low titers are found in normal individuals, which explains the difficulty in defining normal values that would meet general agreement. More important is the fact that no evidence has been advanced as to the clinical role of antiglobulin reactivity. There is an obvious potential for autoimmune stimulation, and clearly, for rapid antibody neutralization by excess of cross-reacting Ig. This situation may conceivably dictate a delicate balance of pathogenetic implications.

The skin window technic has been widely utilized for monitoring leukocyte reticuloendothelial function

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**Table 2. Estimated Immunogenic Lymphocytosis†**

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<th>Lymphocyte Counts</th>
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<tr>
<td></td>
<td>3 hours</td>
</tr>
<tr>
<td>DPT–Saline Controls (n = 10)</td>
<td>15 ± 8.69</td>
</tr>
<tr>
<td>H-IgG-Saline Controls (n = 10)</td>
<td>6 ± 2.33</td>
</tr>
<tr>
<td>APSGN (n = 20)</td>
<td>15 ± 15.39</td>
</tr>
<tr>
<td>RA (n = 9)</td>
<td>−4 ± 4.42</td>
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† The estimated immunogenic lymphocyte response was calculated subtracting the lymphocyte counts obtained in the skin window with saline solution from those obtained in the paired skin window tested with human IgG (H-IgG) or Diphteria-Tetanus-Pertussis (DPT) antigen.

* Significant difference (p < 0.05 or less) between the control group and the patients in the H-IgG-Saline category. APSGN = poststreptococcal glomerulonephritis; RA = rheumatoid arthritis.
in man. The immune response to DPT antigens to which the individual had been prophylactically exposed has been well defined. Briefly, at three hours, the cellular migration consists predominantly of neutrophils, with occasional monocytes, roughly in the same concentration found in the peripheral blood. Lymphocytes start migrating in small numbers at six hours and they have small size (6–8 μm in diameter) that contrasts with that of the monocytes (about 20 μm in diameter). The number and size of the migrating lymphocytes increase progressively with time. At 14 hours, the lymphocyte size is comparable to that of the early appearing monocytes. Prior exposure to the antigen is usually considered a prerequisite for lymphocyte migration to the skin window. The majority of the mononuclear cells have been transformed to the macrophage state by the 24th hour of the study. This sequence of events was observed in controls with DPT antigen and in almost half of the patients with H-IgG.

The data collected in our patients has a great deal of individual variability. It may be appreciated in Table 1, that the standard error of the mean approached the mean cellular counts in many instances. Therefore, mean lymphocyte counts after nine hours were not found to be statistically significant, even though they were several times higher than those of controls with H-IgG reagent. In order to evaluate the problem more critically, it is necessary to consider whether or not the observed differences could be due to variations in the technique of preparation of the skin window. This does not appear to be the case, since skin windows prepared in the same person and tested with the same reagent gave results which were within 15% of each other (see methods). It is then pertinent to analyze the results individually and in addition, to compare the reaction obtained with H-IgG and DPT with the reaction to the saline solvent applied to the opposite forearm. The individual results (Fig. 1) are important because they show that 45% of the patients with poststreptococcal glomerulonephritis and four out of nine patients with rheumatoid arthritis had lymphocyte counts with H-IgG which were more than two SD above the mean response of controls. When the lymphocyte migration was studied subtracting the migration obtained with saline in each individual (Fig. 2), the results are similar. Furthermore, it can be appreciated in Table 2, that the mean lymphocytosis observed in the patient groups attributable to H-IgG reactivity (estimated immunogenic lymphocytosis), is comparable to that found in control subjects challenged with DPT antigen and significantly higher than the normal reaction to the H-IgG reagent (Table 2). Our results are expressed as lymphocyte counts instead of percent of the total cellularity because of a better idea of the magnitude of lymphocyte migration is obtained with absolute numbers. Calculations performed with percent values gave essentially the same results.

There was no demonstrable correlation between the rheumatoid factor titers and the skin window reactivity to H-IgG. This is not surprising since it is not expected that the levels of circulating antibody would be the determining factor in the recognition of an antigen applied to the skin. Serum C3 levels were depressed in the majority of patients with nephritis since most were tested within the first four weeks of the disease. Gewurz and co-workers have reported that patients with acute glomerulonephritis have a heat-labile plasma factor that inhibits the chemotaxis and neutrophil exudation to the skin. Their studies, however, concerned only the response to scratching without antigenic stimulation and, therefore, are not comparable to our investigation.

It is conceivable that cell migration to the skin window could be due to non-specific mechanisms such as activation of complement by aggregation of IgG and endotoxin in the DPT preparation. If such were the
case one would have to postulate a hyperreactivity in the patient population with respect to controls since the latter had lesser lymphocytic responses with IgG. Non-specific hyperreactivity in the patients is unlikely in view of the fact that the patients had similar responses with DPT as did control subjects.

From the data presented, it would appear that the patients with rheumatoid arthritis and poststreptococcal glomerulonephritis may respond to IgG in the skin window in a manner that suggests antigenic recognition. The ultimate significance of this finding is the pathogenesis of these diseases remains to be determined.

References