A Novel Methodology for Quantitating the Enhancement of Cutaneous Delayed-Type Hypersensitivity by IMREG-1: A Measure of the Immunopotentiation of Cell-Mediated Immunity

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INTRODUCTION

Previously we have described the purification and immunomodulatory activities of IMREG-1, a low-molecular-weight subfraction derived from normal human leukocyte dialysates (1-7). This endogenous immunomodulator is recovered from freeze-dried dialyzable leukocyte extracts that are subjected to a series of high-pressure liquid chromatography (HPLC) purification procedures (1). Analysis of IMREG-1 has revealed the presence of two immunologically active peptides, tyrosine-glycine-glycine (Tyr-Gly-Gly) and tyrosine-glycine (Tyr-Gly), as well as the amino acid phenylalanine (Phe) which lacks immunological activity (1). We have noted that these peptides are identical to the amino terminal residues of the enkephalins, and thus may play an important role in interactions between the immune and neuroendocrine systems (1, 8). The tripeptide (Tyr-Gly-Gly) is present in IMREG-1 at approximately 10- to 20-fold less concentration than the dipeptide (Tyr-Gly) (7).

Several immunomodulatory activities of IMREG-1, Tyr-Gly and Tyr-Gly-Gly have been described both in vivo and in vitro (1, 4-6, 8, 9). These include enhancement of the production of IL-2 and interferon-γ (IFN-γ), by normal peripheral blood mononuclear cells (PBMC) stimulated in vitro with mitogen or recall antigens (1, 4) as well as heightened expression of the IL-2 receptor on CD4+ cells exposed to a recall antigen, tetanus toxoid (9). Mitogen-stimulated PBMC from HIV-infected donors have also shown concentration-dependent enhancement of IL-2 production in response to IMREG-1 (4). The modulation of several other in vitro responses have been demonstrated which further illustrate the important effects of these small peptides on the immunoregulatory system (6, 8).

Earlier studies had demonstrated the concentration-dependent enhancement by IMREG-1 of cutaneous delayed-type hypersensitivity (DTH) responses to recall antigens. In these studies, various dilutions of either...
IMREG-1, Tyr–Gly–Gly (YGG) or Tyr–Gly (YG) were mixed with suboptimal amounts of antigen to which normal human subjects had displayed prior sensitivity and then injected intradermally into the volar surface of the forearm. At particular concentrations, each preparation resulted in an accelerated and intensified response as measured by the size of erythema and induration in the presence of antigen (1–5). Neither 1:1 or 1:2 mixtures of the single amino acids L-Tyr and L-Gly had any affect, and there was no response to IMREG-1 in the absence of antigen (1, 2).

Delayed-type hypersensitivity, as determined by cutaneous skin responses, has been a well accepted measurement of cell-mediated immunity for nearly 50 years (10, 11). In a previous communication, we briefly referred to a methodology for the measurement of IMREG-1 enhanced DTH responses (5). Here, we describe in detail this useful approach and methodology for quantifying and comparing cutaneous skin test responses. These methods make use of the acceleration and enhancement of erythema to quantitate the response over time. Furthermore, we can elucidate the time period during which this enhancement occurs and demonstrate that the effect of the immunomodulators is on delayed-type hypersensitivity. Erythema is an early phase of DTH and, although all of its physiological characteristics are not well defined, results directly or indirectly from cytokine release (12–14). Although induration was recorded, measurement of erythema was preferable over induration in that erythema preceded the lymphocytic infiltration and deposition of fibrin responsible for induration (12, 13) and was more accurately measurable than induration. Therefore, this methodology using erythema should be useful in future studies to analyze the activity of IMREG-1 as well as other immunomodulators that enhance DTH responses.

MATERIALS AND METHODS

Preparation of IMREG-1. Procedures for the isolation and purification of IMREG-1 have been previously described in detail (1–7). Briefly, IMREG-1 was prepared from normal human leukocyte extracts via sequential dialysis and two-step reverse-phase high-pressure liquid chromatography (HPLC) fractionation. After collection of the appropriate peaks, the material was aliquoted, freeze-dried, and stored at −70°C until use (1, 7). Four preparations of IMREG-1, which were derived from leukocyte extract preparations that ranged from 125,000 to 400,000 cell equivalents in 1 µl of undiluted neat material and had been shown to have similar activity in preliminary skin test screens, were used in these studies.

Preparation of placebo. Two placebo preparations were used in these studies for control purposes. One was a combination of the single amino acids L-tyrosine and L-glycine in a 1:1 ratio while the other was purified phenylalanine (Phe). Both were purified via HPLC fractionation and collected as previously described. After adjustment to concentrations comparable to that which would be present in IMREG-1, the placebo preparations were freeze-dried and stored at −70°C. As both of these preparations have been previously shown to lack activity in the skin test assay (1), they were collectively referred to as “placebo” in these studies.

Skin test procedure. Frozen aliquots of IMREG-1 or placebo were reconstituted with sterile saline (sodium chloride, injection, USP, LyphoMed Inc. Rosemont, IL) and then serially diluted with sterile saline into tubes (Falcon, Becton Dickinson, Lincoln Park, NJ). Tetanus toxoid, fluid (Connaught Laboratories, Swiftwater, PA), a recall antigen to which the recipients had shown prior sensitivity, was then diluted into sterile saline to a predetermined concentration sufficient to permit modulation of the skin test response. Allergist syringes (0.5 ml with 27-gauge 3/8 intradermal bevel needles, Becton Dickinson, Rutherford, NJ) were filled with antigen plus saline (tetanus toxoid antigen alone control) or antigen plus the dilution of either IMREG-1 or placebo. Subjects were then injected intradermally with 0.15 ml of the mixture into the volar surface of the forearm.

Each test site was scored for erythema and induration at approximately 6, 12, and 24 hr after injection. Erythema and induration were measured by recording the longest and midpoint orthogonal diameters in millimeters (15). Only the erythema measurements were used for the calculations described below.

Before skin testing, all recipients provided written informed consent and all protocols were approved by the Tulane University School of Medicine Institutional Committee on Use of Human Subjects in Research (IRB).

Calculations. The longest (d₁) and midpoint orthogonal diameter (d₂) of erythema were used to calculate the area of erythema. As the response most closely approximated an ellipse, the area of erythema was calculated according to the formula

\[ A = \frac{1}{2} d_1 \frac{1}{2} d_2 \pi, \]

where \( A \) is the area of erythema and \( d \) is the diameter.

The area of erythema was then plotted as a function of time, and the area under the resulting curve (AUC) was calculated for these data by use of the trapezoidal rule (16). The area of a trapezoid, formed by each sequential pair of time points, was determined by the formula

\[ \text{Area under the curve (AUC)} = \frac{1}{2} (A_1 + A_2) (t_2 - t_1), \]

where \( A \) is the area of erythema and \( t \) is time. The total AUC for a given test site is the sum of the segments.
under the curve between each time of measurement. The AUC was calculated at the indicated time points for each dilution or control site, giving an overall measure of response at each test site.

The AUC for each dilution of IMREG-1 or placebo was then compared to each control (antigen alone) by means of a ratio. The AUC for each test site (IMREG-1 or placebo) was divided by the AUC for the respective control site (antigen alone) for each individual recipient. This test/control ratio, i.e., T/C ratio, thus served as a means of quantitating the enhancement of erythema by the test material plus tetanus toxoid compared to the site receiving tetanus toxoid alone. In order to demonstrate the enhancement of the DTH response by IMREG-1, the mean T/C ratios for the same dilution (and same position on the forearm) were compared for the group of subjects who received IMREG-1 plus antigen vs the group of subjects who received placebo plus antigen.

A corrected T/C ratio was also calculated to control for any potential positional effects (i.e., one part of the forearm showing an intrinsically higher capacity to respond to antigen). The corrected T/C ratio was the mean T/C of the group of subjects who received IMREG-1 plus antigen divided by the mean T/C of the group who received placebo plus antigen at the same dilution. Because the same dilutions for IMREG-1 plus antigen and placebo plus antigen were injected in the same order and at comparable positions on the volar surfaces of each individual's arm, the corrected T/C ratio takes into account any potential positional bias that might be introduced.

**Statistical analysis.** To assess the difference in mean response for each individual dilution in the group receiving IMREG-1 plus antigen, compared to that dilution in the group receiving placebo plus antigen, the Mann–Whitney test (one-tailed) was used. The overall increase in response in the group receiving IMREG-1 plus antigen, compared to placebo plus antigen, was assessed by ANOVA analysis. Statistical analyses were performed using BMDP PC-90 software (BMDP Statistical Software, Inc., Los Angeles, CA).

**RESULTS AND DISCUSSION**

Although IMREG-1 has been shown to modulate skin test responses to several recall antigens including purified protein derivative (PPD), histoplasmin, and streptokinase/streptodornase (1–5), we selected tetanus toxoid for these studies due to the larger pool of available subjects who respond to this antigen. Skin test screens with tetanus toxoid alone indicated the degree of sensitivity for each individual recipient and permitted the selection of an appropriate concentration of antigen to be used for that subject in the subsequent skin test with either drug or placebo. Suboptimal concentrations of antigen were chosen that at control (tetanus toxoid alone) sites yielded a sum of the longest and orthogonal diameters of erythema approximating 10–16 mm at either 12 or 24 hr after testing. The skin test screens had only a negligible effect on the response to tetanus toxoid alone in subsequent skin tests and any slight boosting effect which may have occurred would be controlled by the methodology described below.

**FIG. 1.** Area of erythema (mm²) versus time for one test subject injected with either a 1/15 dilution of tetanus toxoid (0.027 Lf) or a 10⁻⁶ dilution of IMREG-1 plus a 1/15 dilution of tetanus toxoid.
In the initial screens of the IMREG-1 preparations, each generally showed peak activity at either a $10^{-1}$ or $10^{-8}$ dilution of neat, undiluted IMREG-1, peak activity being a maximum increase in erythema. Thus, concentrations from $10^{-8}$ to $10^{-9}$ were examined using 29 healthy test subjects who were each sensitive to tetanus toxoid; 20 subjects received IMREG-1 and 9 received placebo.

Figure 1 illustrates a representative plot of area of erythema versus time for one test subject. As demonstrated in this figure, the presence of IMREG-1 accelerated and enhanced the response to antigen alone. Although the greatest acceleration of the response occurred between 6 and 12 hr, in terms of percentage increase in the area of erythema, the response continued to persist and increase between 12 and 24 hr. Thus, as a function of time, the response occurred predominantly in the 12- to 24-hr time interval. As is characteristic of DTH, the response continued beyond 24 hr. The period up to 24 hr was selected, since beyond that time the response of the individual test site would be more likely to be influenced by local diffusion and other factors.

We used the methodology of calculating the AUC in order to capture the sustained enhancement over time characteristic of the DTH response. The curve plots erythema as a function of time. This approach, based on the trapezoidal rule wherein concentration—time curves are converted to a series of trapezoids, is commonly used to determine various pharmacokinetic parameters such as bioavailability (16). Thus, using this calculation with area of erythema makes full use of the activity of the drug and its ability to accelerate and enhance erythema over time. It also provides a means by which this response can be quantitated.

In the example shown in Fig. 1, the AUCs for antigen alone corresponding to the time periods from 0 to 6, 6 to 12, and 12 to 24 hr were 3, 24, and 750, respectively. The AUCs for antigen plus a $10^{-8}$ dilution of IMREG-1 were 213, 894, and 3330 for 0 to 6, 6 to 12, and 12 to 24 hr, respectively. The total AUC for antigen alone was 777, whereas the total AUC for antigen plus IMREG-1 in that interval was 4437. Thus, the response can be expressed quantitatively and further demonstrates that enhancement of erythema by IMREG-1 continued to accelerate and persist predominantly during the 12- to 24-hr time frame. The responses to antigen alone and placebo plus antigen also occurred predominantly during the 12- to 24-hr period (data not shown).

The AUCs were then used to calculate the T/C ratio such that the AUC for each test site was divided by the AUC for the respective control (antigen alone) for that individual. This provided an internal control for any individual variation between subjects, and reduced the data to a parameter that permitted a ready comparison of the response to drug and placebo. The closer a T/C ratio is to 1.00, the more the response to a particular dilution of drug or placebo approaches the response of antigen alone.

In earlier reports, we noted that IMREG-1 does not enhance any response to an antigen to which the recipient does not show prior sensitivity, thus distinguishing this product from classical "transfer factor" (2, 3). It is also noteworthy that in a normal nonimmunocompromised human population, not all skin test recipients will manifest amplification of response to an antigen which they had been previously sensitized. The reasons for this "nonresponse" have not been elucidated, but may be related to the presence or absence or state of activity of particular key immune cells and complex regulatory characteristics of the immune system itself. For example, if the amount of antigen used in the skin test is too high and essentially induces a maximum response, then no amplification will occur since an immunomodulator would not be expected to enhance a response beyond capacity. Thus, subjects were prescreened for their degree of sensitivity to antigen, in order that suboptimal concentrations of tetanus toxoid could be used in the present study.

In Table 1, the mean T/C ratios for IMREG-1 plus antigen ($n = 20$) and placebo plus antigen ($n = 9$) are shown for the time period from 0 to 24 hr. The overall increase in response of IMREG-1 compared to placebo was significant ($P < 0.001$) by ANOVA. The response to IMREG-1 plus antigen was statistically significant compared to that of placebo plus antigen at dilutions of $10^{-7}$, $10^{-8}$, and $10^{-9}$.

In Table 2, the mean T/C ratios for IMREG-1 plus antigen are shown for the 12- to 24-hr period. ANOVA analysis of the overall increase in response was significant and the responses expressed by use of the T/C ratio at dilutions of $10^{-7}$, $10^{-8}$, and $10^{-9}$ of IMREG-1 were significantly different from those for placebo.

In each table, the corrected T/C ratios are also shown for the appropriate time periods. The corrected T/C ratio takes into account any possible positional effects similar to those that have been described by others (17, 18). Since the mean T/C ratio of IMREG-1 plus antigen divided by the mean T/C ratio of placebo plus antigen at the same dilution (and position on the forearm) is considerably more than 1.00, the effect of the drug at any concentration cannot be attributed to an area of

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Mean Test/Control (T/C) Ratio by Test Material and Dilution over the 0- to 24-hr Time Period</th>
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<tbody>
<tr>
<td></td>
<td>Dilution of test material</td>
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<tr>
<td></td>
<td>$10^{-6}$</td>
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<tr>
<td>IMREG-1 ($n = 20$)</td>
<td>1.86</td>
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<tr>
<td>Placebo ($n = 9$)</td>
<td>1.17</td>
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<tr>
<td>$P$ (Mann–Whitney)</td>
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<tr>
<td>ANOVA $F = 24.87$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Corrected T/C</td>
<td>1.59</td>
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the forearm having an intrinsically higher response than that to antigen alone.

It is important to note that in this initial study, only limited dilutions of IMREG-1 were examined. Thus, the range of activity of IMREG-1 cannot be completely elucidated from these data. For example, the response to a $10^{-6}$ dilution of IMREG-1 was not significant and thus may represent a point which is not at optimal concentration. At $10^{-7}$, $10^{-8}$, and $10^{-9}$ dilutions of IMREG-1, the response appears to essentially plateau. However, in studies to be reported at a later date, we have noted that this response decreases as the immunomodulator becomes more dilute. Although the present studies were performed without randomization or binding, further skin tests examining this activity are being conducted using randomized double-blind, placebo-controlled protocols.

Table 2 also illustrates the percentage of total (0 to 24 hr) response, as measured by AUC, that occurred in the 12- to 24-hr period. As shown, at each dilution of either IMREG-1 or placebo, the majority of the reaction occurred during the 12- to 24-hr time period. For example, with subjects receiving IMREG-1 plus antigen ($n = 20$) an average of approximately 65% of the reaction occurred during the 12- to 24-hr time period, 25% occurred during the 6- to 12-hr period, and approximately 8% from 0 to 6 hr. In the placebo groups, approximately 80% of the reaction occurred during the 12- to 24-hr period, approximately 16% from 6 to 12 hr, and approximately 4% from 0 to 6 hr. The fact that slightly more of the response occurred in the 6- to 12-hr time frame in the IMREG-1 plus antigen group over the placebo plus antigen group may also be indicative of the ability of IMREG-1 to accelerate and then also sustain the response.

We feel that this acceleration and enhancement of erythema and the sustained response during the 12- to 24-hr interval indicate an upregulation of cell-mediated immunity. Although a very small Arthus reaction may contribute to the response, the observations reported here are almost entirely attributable to DTH. First, Arthus reactions generally occur between 2 and 6 hr and disappear within 12 hr (20-23). Furthermore, they are described as being soft and boggy (edematous) and are characterized by neutrophil infiltration (20-22). DTH reactions to recall antigens, on the other hand, are indurated due to mononuclear cell infiltration and deposition of fibrin and occur a few hours later (13, 19). In addition to the induration reported in our studies, punch biopsies of the IMREG-1 and antigen sites in past studies clearly demonstrated mononuclear cell infiltration without evidence of neutrophils in perivenular areas (2). Biopsies of sites receiving antigen alone in those studies demonstrated a mild lymphocyte vasculitis consisting of perivascular infiltrates of lymphocytes and some neutrophils (2). Since an influx of basophils was not noted in these previous biopsies, it is doubtful that this response is due in a large extent to a Jones–Mote reaction (also mediated by antigen-stimulated T cells and thus cell-mediated) although a very small amount of basophils may contribute to the overall effect (24). As has been noted by others, the small amount of antigen used in these studies also favors the elicitation of DTH reactions as opposed to Arthus (23). Thus, the responses reported here appear to be predominantly cell-mediated and one of delayed-type hypersensitivity in contrast to an antibody-mediated phenomenon.

These results, along with previously described immunomodulatory activities of IMREG-1, provide further insight into the overall dynamics of how this immune regulator is able to modulate various facets of the immune system (1-9). Ultimately, the enhanced and sustained erythema in these studies is most likely the result of modulated cytokine production leading to increases in extravasation, the recruitment of mononuclear cells, and the eventual activation of macrophages and other immune cells (13, 19). The enhanced in vitro production of IL-2, IFN-γ, and MIF by IMREG-1 that we have previously observed supports this view. However, the effect of the drug may be due not only to the increase in number of infiltrating cells but also in the possible enhanced cytokine output of these cells once they arrive at the site. Overall, the effect is one of enhanced cell-mediated immunity leading to increased cytokine output and the resulting erythema.

In this study we describe a new methodology for analyzing the skin test response to IMREG-1. This has enabled us to describe and quantitate the differences between the response to IMREG-1 plus antigen in comparison to the response to placebo plus antigen. These same techniques could also have an application with other modulators of DTH responses and possibly in other cases where measurement of erythema is undertaken. DTH is an important functional measure of cell-mediated immunity not only in normal individuals, but also in those who are immune deficient, such as in HIV disease (25-27). Thus, this methodology for quantitative assessment of a DTH response will be useful for future determination of the biological activity of IMREG-1 preparations. It may also prove generally useful in assessment of other immunomodulators and
ENHANCEMENT OF DTH BY IMREG-1

quantitation of similar immunologic responses. We would also note that as a result of these studies, we have shown that IMREG-1 is capable of enhancing human DTH which is a direct manifestation of cell-mediated immunity. We believe it reasonable to suggest that IMREG-1 could be useful therapeutically in conditions in which cell-mediated immunity is impaired.

REFERENCES


