Enhancing Activity of Various Immunoaugmenting Agents on the Delayed-Type Hypersensitivity Response in Mice

Anna Bartocci, Elizabeth L. Read, Roy D. Welker, Erich Schlick, Vasilios Papademetriou, and Michael A. Chirigos

Laboratory of Chemical Pharmacology, National Cancer Institute, NIH, Bethesda, Maryland 20205

ABSTRACT

Six immunoaugmenting agents were tested in the delayed-type hypersensitivity reaction (DTH) in normal BALB/c × DBA/2 mice. The agents tested, levan, lentinan, mannozym, maleic anhydride divinyl ether, polyriboinosinic-polycytidylic acid-poly-L-lysine, and highly purified L-cell interferon, gave significant increases in the DTH response above the sheep red blood cell control. The schedule of doses for each agent corresponded with previous experiments from this laboratory of the maximum natural killer cell activity, macrophage activation, and interferon induction. Highly purified L-cell interferon was capable of eliciting a significant DTH response when given 4 hr after the initial challenge with sheep erythrocytes. In addition, λ-carrageenan, a macrophage-cytotoxic agent which can render the macrophage inactive, was found to suppress the DTH response to levels slightly above phosphate-buffered saline controls. The carrageenan-induced suppression of the DTH response could be abrogated by coadministration with immunoaugmenting agents to levels attained with the immunoaugmenting agents alone.

INTRODUCTION

The DTH response is considered to result from interaction in vivo between macrophages presenting foreign antigens to the T-cells. Macrophages can also affect T-cell function in the DTH response by releasing soluble factors that enhance development of precursor cells into effector T-cells.

In the final step(s), the lymphokines formed by the antigen-stimulated T-cell activate the macrophages which participate in the DTH response (13, 20, 23). The histological evidence for the presence of mononuclear cell infiltration (primarily lymphocytes and macrophages) at the site of challenge supports the fact that both lymphocytes and macrophages participate in the DTH response (9).

Several agents have been reported to exert a regulatory effect on various cellular components of the immune system. IFs have been reported to increase phagocytosis (15), enhance macrophage tumoricidal activity (25), and augment NK cell activity (11, 12, 31). Type I IF has also been described as influencing cell-mediated immunity (8) and the afferent (9, 10) and efferent (8) pathways of the DTH response. Other agents have been reported to have an effect on one or more cellular elements of the immune system (1, 3–6). Since the DTH response is a measure of both macrophage and T-cell interaction, it was of interest to assess whether a correlation exists between the capacity of selected agents which enhance macrophage and/or NK cell activity and their ability to effect the DTH response. The role of the macrophage in the DTH response would be determined by using CARR to suppress macrophages (2, 24, 32).

This report presents the results of testing 3 polysaccharides (levan, lentinan, mannozym), 2 chemicals (poly ICLC, MVE-2), and IF for their capacity to enhance the DTH response to SRBC and to overcome CARR-induced suppression of this response.

MATERIALS AND METHODS

Animals. Adult male 6- to 8-week-old BALB/c × DBA/2 (hereafter called CD2F1) weighing approximately 25 g were supplied by the Mammalian Genetics and Animal Production Section, Drug Research and Development, National Cancer Institute, NIH, Bethesda, Md. Mice were housed in plastic cages with air filter bonnets and fed Purina laboratory chow and water ad libitum.

Drugs. Levan, a polysaccharide extracted from the cell wall of Aerobacter levan, was generously supplied by Dr. M. Walman, Department of Pathology, Tel Aviv University Medical School, Tel Aviv, Israel. Lentinan, a β-1,3-glucan obtained from the mushroom Lentinan edodes, was a gift from Dr. Yasumi Yugari of the Life Science Laboratory, Ajinomoto Company, Yokohoma, Japan. Mannozym, a glucomannan polysaccharide purified from Saccharomyces cerevisiae, was generously provided by Dr. Pierre Jacques, Biomedical Cytology, Centre Interuniversitaire de Recherches Multidisciplinaires en Affections Parasitaires et Mecanismes de Defense de l’Hote, Brussels, Belgium. Levan, lentinan, and mannozym were tested for the presence of endotoxin, and none could be detected (14). MVE-2 was supplied by Dr. Richard Carrano, Adria Laboratories, Columbus, Ohio. Poly ICLC stabilized with carboxymethylcellulose was obtained from Dr. Hilton Levy, Molecular Virology Section, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md. Highly purified mouse L-cell IF was purchased from Dr. Kurt Paucker, University of Pennsylvania Medical School, Philadelphia, Pa. CARR was purchased from Sigma Chemical Company, St. Louis, Mo., and was dissolved in PBS by heating for 10 min.

DTH Assay. Fresh sheep erythrocytes purchased from Flow Laboratories, McLean, Va., were washed 3 times in PBS and diluted to 2 × 10⁸ cells/ml. The left rear footpad of CD2F1 mice was given i.d. injections of 0.05 ml of the SRBC suspension on Day 0. On Day 5, 0.05 ml of SRBC at 2.0 × 10⁹ cells/ml was injected i.d. into the right rear footpad. Three measurements of each rear footpad were taken with a gravity-sensitive calibrated measuring device on Day 5. The difference in footpad swelling between the right (sensitized) rear and left rear footpad was recorded as the individual DTH response. Each experimental group contained 8 mice. A PBS (negative) control and a SRBC (positive) control were also included in each experiment. The standard deviation within each group never exceeded ±0.03 mm.

Received August 17, 1981; accepted May 28, 1982.
Significant values for the experimental groups were obtained by using the Student's t test.

**Macrophage Activation Assay.** The assay for measuring the activity of agents to induce macrophage-mediated cytotoxicity has been described previously (27). Briefly, 6- to 8-week-old BALB/c mice received a single i.p. treatment of drugs or PBS. Peritoneal macrophages were harvested, adjusted to 8.0 \( \times 10^6 \) macrophages per ml in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum and 125 \( \mu \text{g} \) gentamicin per ml, and allowed to adhere for 2 hr in 16-mm wells (tissue culture cluster plate; Costar, Cambridge, Mass.). After a washing to remove nonadherent cells, the macrophage monolayers were overlaid with MBL-2 leukemia cells adjusted to 8.0 \( \times 10^5 \) cells/ml. All cultures were maintained in a humidified incubator with 5% \( \text{CO}_2 \)-air at 37°. Viable MBL-2 cells were counted in a hemocytometer after 48 hr. Percentage of growth inhibition of MBL-2 cells due to macrophage activation was calculated by comparison to MBL-2 cells grown in the presence of normal control macrophages.

**NK Cell Assay.** A conventional \( ^{51} \text{Cr} \) release assay was used as described previously by others (16). In brief, 2.0 \( \times 10^6 \) radiolabeled YAC-1 (7) target cells (100 \( \mu \text{Ci} \) of \( ^{51} \text{Cr} \); specific activity, 250 to 800 mCi/mg/1.0 \( \times 10^7 \) tumor cells at 37° for 45 min) in 0.1 ml volume were added to graded numbers of splenic effector cells (final effector:target ratios were 200:1, 100:1, 50:1, or 25:1) in round bottomed 96-well microtiter plates. The final volume was 0.2 ml/well. Triplicate cultures were maintained in a humidified incubator, 5% \( \text{CO}_2 \)-air incubator at 37° for 4 hr. The plates were then centrifuged for 10 min at 800 \( \times g \), and a volume of 0.1 ml of supernatant was removed and measured in a gamma counter. Spontaneous release was determined by incubation of 2.0 \( \times 10^6 \) tumor cells in 0.2 ml of Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum and 125 \( \mu \text{g} \) gentamicin per ml. Maximum release of radioactivity was determined by freezing and thawing of 2.0 \( \times 10^4 \) tumor cells 4 times in an acetone/dry ice bath. Maximum release of radioactivity varied from 85 to 95% of total isotope uptake, and spontaneous release varied from 7 to 12% of maximum release. The percentage of cytotoxicity was calculated from the formula:

\[
\text{Experimental cpm} - \text{spontaneous release cpm} \\
\text{max release cpm} - \text{spontaneous release cpm} \\
\% \text{of cytotoxicity} = \frac{\times 100}{100}
\]

**RESULTS**

**Effect of Test Agents on Macrophage and NK Cell Activity.** All the agents were found to be effective in enhancing macrophage tumoricidal activity (Table 1). The optimum response occurred at different time intervals after injection of test agent. The peak of activity for levan and lentinan occurred at 6 days after injection, for mannozym and MVE-2 at 3 days, and for poly ICLC at 1 day.

The most significant augmentation of NK cell activity paralleled the same time as that observed on macrophage activation for mannozym, MVE-2, and polyICLC. However, the peak of NK cell activity for levan and lentinan occurred 3 days prior to their peak of macrophage activation.

**DTH Response.** The test agents were examined for their capacity to enhance the DTH response. Each agent was administered at various times prior to the initial SRBC sensitization (Table 2). The results of several tests show that each agent significantly enhanced the DTH response in a dose-dependent manner (Table 3). Optimum activity for each agent was achieved when it was administered at the same time found to give maximum macrophage activity.

Since poly ICLC was the most effective in enhancing both the macrophage and NK cell activity as well as being the most effective inducer of IF (3, 5, 21), this agent was studied more...
extensively (Chart 1). Significant enhancement of the DTH response occurred when poly ICLC was administered as early as 3 days before and at the time of the initial injection with SRBC. The most significant enhancement occurred when it was given 1 day prior to sensitization. These results correlate well with its optimal effect on macrophage and NK cell response (Table 1). However, if poly ICLC was administered after the initial SRBC sensitization, no effect was seen except when the drug was given on the third day after initial sensitization and a significant depression on the DTH was observed.

**Depression of the DTH Response by CARR.** Various concentrations of CARR have been reported to be capable of depressing the DTH response. First, it was necessary to determine the maximum tolerated dose of CARR. Mice were given i.p. injections of a single dose of CARR ranging from 2 to 40 mg/kg. Mice tolerated the 20-mg/kg dose well. Doses greater than 32 mg/kg result in anorexia and early death. Results in Table 4 show that at the 20-mg/kg CARR dose the maximum depression of the DTH response occurred when administered 2 days prior to SRBC sensitization. When CARR was administered 1 or 2 days after sensitization, little or no depressive effect was observed.

**Capacity of Test Agents to Restore CARR Depression of the DTH Response.** CARR was injected on the day of its most suppressive effect (Day —2), and the test agents were given on the day which produced maximum activity (levan, lentinin, Day —6; mannozym, MVE-2, Day —3; and poly ICLC, Day —1). All of these injections were given prior to the initial challenge with SRBC. All 5 agents effectively reversed the depression of the DTH response induced by CARR treatment (Chart 2). MVE-2 and poly ICLC appeared to be the most effective since they gave a response higher than the SRBC control value alone.

Results in Chart 3 show the mean of the responses of mice when IF was tested for its capacity to reverse CARR depression of the DTH response. CARR administered 2 days prior to SRBC sensitization depressed the response from the SRBC control value of 0.25 to 0.05 mm. IF alone, when administered 4 hr after sensitization, significantly enhanced the DTH response to a value of 0.42 mm. IF was found to be capable of reversing
the CARR-induced depression attaining a value of 0.40 mm, close to that achieved when IF alone was used.

**DISCUSSION**

All 5 agents examined (levan, lentinan, mannozym, MVE-2, and poly ILC) were shown to enhance markedly the response of macrophages and NK cells. Using the same time sequence found optimal for each agent to stimulate macrophage activation, all 5 drugs also significantly enhanced the DTH response. Several studies show that IF plays a regulatory role in macrophage enhancement (26, 28) and NK cell augmentation (11, 12, 31) and, indeed, indicate that IF is the primary signal for regulation of these cellular elements of the immune system. IF, when injected exogenously or induced endogenously by Newcastle Disease Virus, has been reported to play a regulatory role in the DTH response (9, 10). These reports demonstrated that IF exerted a modulatory effect on DTH, inhibiting or enhancing the response in relation to the time of injection. Optimal enhancement with IF occurred when it was injected 4 hr after primary sensitization with SRBC.

Results of the present study show that a dose-dependent enhancement of the DTH with levan, lentinan, mannozym, MVE-2, and poly ILC occurred when they were administered before the primary sensitization with the antigen. It was of interest to speculate whether the enhanced DTH response achieved with these drugs could be due to IF induction and/or due to their direct effect on macrophage activation.

The anionic polymer MVE-2 and polysaccharides (levan, lentinan, and mannozym) appear to be low inducers of IF (3, 21). The enhanced macrophage activation reported to occur with anionic polymers was shown to be completely neutralized by anti-IF globulin indicating that macrophage-induced IF was responsible for the enhanced macrophage activity (26).

Poly ILC has been reported to induce a high titer of IF within 4 hr after injection and remains elevated in the serum for 3 days (5, 18, 22). In contrast to the report that IF injected prior to SRBC sensitization inhibits DTH (9), present results show that the DTH response was augmented when poly ILC was administered 3 days before or on the day of sensitization. Since endogenous poly ILC-induced IF is present in serum for at least 3 days (i.e., at 24 hr, 6300 units/ml; at 48 hr, 1250 units/ml; at 72 hr, 15 units/ml) (5, 18, 22), the high level of poly ILC-induced IF still present when the primary antigen was administered did not inhibit the DTH response. The depression occurring when poly ILC was administered 3 days after primary sensitization (1 day prior to rechallenge) correlates well with the previous observation of DTH suppression using Newcastle Disease Virus as an IF inducer (9).

Several reports have described the suppressive effect exerted by CARR on cellular immune function (2, 17, 19, 30, 32). The immunosuppressive effects after CARR treatment are often attributed to macrophage injury. Treatment of experimental animals with CARR leads to a significant suppression of the DTH response (29). Using CARR in conjunction with IF and the 5 drugs, we were able to reverse the suppressive effects found on the DTH response with CARR alone. The ability of each test agent to abolish the CARR-induced suppression appears to correlate with their capacity to activate macrophages. Levan, lentinan, mannozym, and MVE-2 were given prior to CARR treatment, suggesting that macrophages once activated are able to maintain their capacity to respond to the SRBC and may be less susceptible to the action of CARR. It also seems very possible that a second population of macrophages not damaged in their function by CARR can be reactive as shown by the restoration of the DTH response with poly ILC and IF, which are injected after CARR. Finally, we cannot exclude the fact that IF alone acts directly as a messenger on the T cells responding to the antigen in the DTH response, thus overcoming the suppressing properties of CARR. The enhanced DTH response obtained with the 6 immunoaugmenting agents correlated well with their capacity to regulate macrophage and NK cell activity. This DTH assay could serve as an additional cell-mediated assay for evaluating potential immunoaugmenting agents.

**ACKNOWLEDGMENTS**

We gratefully acknowledge M. R. Mitchell for her excellent technical assistance and C. Bower for her excellent secretarial assistance.

**REFERENCES**


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