Journal of Medical Virology 34:68–73 (1991)

Effect of Rimantadine on the Immune Response to Influenza A Infections

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The effects of rimantadine on lymphocyte responses to mitogens CON-A and PHA, natural killer cell activity, and the development of serum and local antibodies were studied during an epidemic outbreak of influenza A (H3N2). Twenty-three families consisting of 38 adults and 46 children had a member who developed a flu-like illness and were randomly assigned to receive placebo or rimantadine either as treatment or post exposure prophylaxis. Nasal washings for virus isolation and IgG and IgA determination were collected on days 1, 5, and 10 of illness. Blood samples for immunologic studies were obtained on days 1 and 5 of clinical illness and on day 21. No differences in lymphocyte responses to CON-A and PHA or in natural-killer cell activity were noted between placebo and rimantadine groups. The development of neutralizing antibodies to influenza H3N2 was also not affected by rimantadine. However, the presence of IgA in nasal secretions was significantly diminished in the rimantadine group compared to the placebo group (0/9 vs. 6/9, $P < 0.005$).

The findings indicate that rimantadine had no adverse affect on the systemic immune system. However, local immune response was diminished in individuals taking rimantadine possibly due to the presence of less immunogen resulting from reduction of virus in secretions of individuals taking antivirals.

KEY WORDS: immunoglobulin, lymphocyte blastogenesis, natural killer cell activity, Rimantadine, influenza A

INTRODUCTION

Influenza continues to be a major cause of morbidity and mortality in the United States [Glezen et al., 1987; Barker et al., 1986; Barker et al., 1980; CDC 1988]. Because of the variable efficacy and utilization of the influenza vaccine, interest in antiviral compounds has increased. Amantadine and its structural analog rimantadine have been shown to be beneficial in both the treatment and prophylaxis of influenza A infections [Dolin et al., 1982; Hall et al., 1987; Van Voris et al., 1981; Clover et al., 1986; Tominack et al., 1987]. However, the potential adverse effect of these compounds on the immune response to influenza A infections has raised concerns about their use.

Only a few authors have reported the effects of rimantadine on the immune response to influenza A infection. An in vitro study which utilized extremely high doses of rimantadine demonstrated that rimantadine may have a immunosuppressive effect on T lymphocytes [Koff et al., 1979]. Herrmann [1990] reported that rimantadine treatment depressed cytotoxic T lymphocyte response and antibody response in a mouse model. The authors concluded that the depressed response was due to a decrease in the amount of antigen available or interference with viral antigen processing.

With the increasing availability of antivirals for the treatment and prophylaxis of influenza A infections, a better understanding of their effect on the human immune system is needed. A naturally occurring epidemic of influenza A H3N2 allowed us to study the effect of rimantadine on certain parameters of the human immune system.

METHODS

Study Design

During a multi-centered trial studying the clinical efficacy of early treatment and secondary prophylaxis with rimantadine in families, we elected to study the effect of rimantadine on the immune system. Details of the methodology for the above mentioned study have been described elsewhere [Hayden et al., 1989]. Fifty families were prospectively recruited for inclusion in this study and monitored for the occurrence of illness caused by influenza A virus. When influenza A virus activity was documented in the community from sur-

Accepted for publication January 29, 1991.

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This study was supported in part by a grant from Hoffman LaRoche and approved by the Institutional Review Board at the Oklahoma University Health Sciences Center.

veillance cultures and if influenzal illness developed in 1 or more family members, all eligible family members, including those who were ill and not ill, were randomly assigned as a block to receive either rimantadine or placebo.

Participants

Eligible families consisted of 2 to 5 members with at least 1 adult (ranging in age from 18 to 75 years) and one child (ranging in age from 1 to 17 years) able to take the study drug. Persons were excluded from participation if they had a history of amantadine hypersensitivity, chronic respiratory disease, severe medical illness, or neuropsychiatric disorder; were pregnant or lactating; had a recently documented influenza A virus infection; or required long-term drug therapy with amantadine or drugs that could interfere with rimantadine or with clinical assessments (e.g., aspirin, tranquilizers, antihistamines, and decongestants). Written informed consent in a form approved by the institutional review board of the Oklahoma University Health Sciences Center was obtained. Families were compensated for participation.

Drug Administration

Rimantadine in tablet or syrup form (for children $\leq$ 9 years) or matching placebo was administered once daily for 10 days. The daily dose was 200 mg for adults and for children over nine or those who weighed more than 30 kg. The dose was 5 mg per kilogram of body weight up to a maximum of 150 mg per day for children $\leq$ 9 years of age or for those weighing less than 30 kg. All eligible family members were given the assigned drug as soon as influenzal illness was first recognized in a family member (the index patient) and after the member had been evaluated by the study nurse.

Surveillance and Sampling

The household was visited by a study nurse within 24 hours after the index case was reported. Serum samples were collected from all participants for later serologic testing, and nasal washings for viral isolation and for detection of IgG and IgA were taken from ill family members. An additional 10 cc of blood was taken from the index cases and a non-ill adult (if available) for lymphocyte studies as described below. Nasal aspirates or nose and throat swabs were sometimes collected instead of washings in children. Symptom records, including afternoon measurements of the oral temperature, were completed for each family member for the 10-day treatment period. If any family member subsequently became ill during the treatment period, specimens were collected for viral isolation. The household was also visited to collect specimens from the index patient and any other symptomatic family members on the fifth and tenth treatment day. Blood samples were collected during convalescence, three to four weeks after the start of treatment.

Infection and Illness

Laboratory-documented infection was defined as isolation of influenza A virus from secretions or as a 4-fold or larger rise in serum titers of neutralizing antibody. An influenzal illness was defined as the presence of an elevated temperature ($\geq$ 38.0°C) and at least one of the following symptoms: cough, sore throat, nasal obstruction, chest pain, myalgia, headache, runny nose, fatigue and malaise. A nonfebrile respiratory illness was defined as the occurrence of two or more of these respiratory symptoms.

Collection of Specimens for Isolation of Virus

Nasopharyngeal (NP) washes and throat swabs were taken from each subject. Approximately 2 ml of Hank's balanced salt solution (HBSS) were introduced into the nasopharynx, aspirated, and the wash placed in transport medium consisting of tryptic soy broth with 0.5 percent gelatin, gentamicin, chloramphenicol, and amphotericin B. The nasopharynx was also swabbed and the NP swab was placed in the same vial as the wash. Throat swabs were placed in separate vials of transport medium. Specimens were transported promptly to the laboratory on wet ice and processed immediately.

Virologic Studies

Specimens were inoculated onto duplicate monolayers of primary rhesus monkey kidney (PRMK) cells. The cultures were maintained on serum-free medium at 35°C and were viewed daily for cytopathic effect. Every two to three days, the cultures were tested for hemadsorption with guinea pig erythrocytes at 4° and 22°C. The identity of virus isolates was confirmed by indirect immunofluorescence using monoclonal antibodies [Waner et al., 1985]. Influenza A isolates were typed by the Oklahoma State Department of Health. First-passage isolates were stored at −70°C for susceptibility testing to rimantadine in previously described enzyme-linked immunosorbent assays (ELISA) [Belshe et al., 1988, 1989]. These assays were performed by Dr. R. B. Belshe's lab at Marshall University, Huntington, West Virginia.

Detection of IgG and IgA Antibodies to Influenza A in NP Washes

IgG and IgA antibodies were detected by a modification of a procedure described by McIntosh, et al. [1978]. Antigen was prepared by infecting PRMK cells with a local isolate of influenza A and applying infected cells to 8 mm circumscribed wells of 8-well glass slides as previously described [Shalit et al., 1985]; antigen slides were stored at −70°C until used. Aliquotes of NP washes frozen at the time of collection at −70°C were thawed and diluted 1:2 in phosphate buffered saline, pH 7.0 (PBS). Each diluted specimen was applied to duplicate antigen wells on 2 slides. Following 30 minutes of incubation at 37°C, fluorescein conjugated anti-IgG or anti-IgA (Tago, Burlingame, CA) were applied to

each slide and the incubation resumed for an additional 30 minutes. Slides were viewed at 400× magnification by epi-fluorescence. Positive and negative controls were performed in each determination.

Preparation of Lymphocytes

Venous bloods were obtained in heparinized tubes and transported to the laboratory within two hours of collection. Peripheral blood lymphocytes (PBL) were obtained following centrifugation on an equal volume of Histopaque (Sigma, St. Louis, MO) for 30 minutes at 750 × g. The lymphocytes were washed three times with HBSS and suspended in RPMI 1640 medium containing no serum or 10 percent fetal calf serum and 50 μg per ml of gentamicin. The viability of the cells in all assays was greater than 95 percent as determined by trypan blue exclusion. Plasma was recovered from the top of the Histopaque and retained for use in the blastogenic assays.

Lymphocyte Blastogenic Assay

Lymphocytes were suspended at a concentration of 5×10^5 per ml in RPMI-1640 medium with 10 percent autologous plasma obtained following the centrifugation of the blood on histopaque. Phytohemagglutinin (PHA) at concentrations of 2 μg, 1 μg, and 0.5 μg per ml, and concanavalin A (Con-A) at concentrations of 20 μg, 10 μg, and 5 μg per ml were incubated with each patients' lymphocytes. One-tenth ml of the lymphocyte suspension was incubated with 0.1 ml of the mitogen concentrations in wells of 96-well round bottom plates. Four wells of each mitogen concentration were prepared and 4 wells of lymphocytes were incubated without mitogen to obtain background activity. Following 48 hours of incubation at 36°C in a humidified CO_2 atmosphere, 1 μCi of ^{3}H.-thymidine was added to each well. The number of radioactive counts incorporated into the acid insoluble precipitate of each well was determined after an additional 20 hours of incubation. The average counts/min for 4 homologous wells was determined; background counts were subtracted from mitogen determinations.

Natural Killer Cell Activity

The method used was essentially as previously described by Waner et al. [1985]. In brief, K562 cells were incubated with 100 μCi/ml of ^{51}Cr for 1 hour at 37°C. The cells were suspended in RPMI 1640 medium with 10 percent fetal calf serum at a concentration of 10^5 cells/ml. Ten thousand cells were incubated with lymphocytes at effector to target ratios of 40:1, 20:1, and 10:1 in triplicate wells of 96-well round bottom well plates. Following 20 hours of incubation at 37°C 0.1 ml of each well was assayed for ^{51}Cr release. Target cells were incubated alone to determine spontaneous release (SR); total counts were determined by lysing target cells with 5 percent Triton X-100 in water. Percent ^{51}Cr release was calculated as follows: percent ^{51}Cr release = [experimental counts released − SR/ Total counts released − SR] × 100. The degree of natural killer cell activity was expressed as lytic units. One lytic unit (LU) was equivalent to the number of effector cells that gave 30 percent lysis of target cells.

Neutralization Test

The neutralization test procedure employed was essentially that described by Frank et al. [1980]. Sera were inactivated at 56°C for 30 minutes. Two-fold dilutions of sera were made in Eagle's minimal essential medium (MEM) and incubated for 60 minutes at room temperature with 2 $TCID_{50}$ of a strain of influenza A isolated during the study and prepared as cell free stock. Duplicate monolayers of MDCK cells in flat-bottom 96-well trays were each inoculated with 0.1 ml of each dilution. Following one hour of adsorption at 37°, the inoculum was aspirated and the cultures refed with serum-free medium. Forty-eight hours later the cultures were observed for cytopathic effect (CPE). The greatest dilution of serum resulting in 50 percent inhibition of CPE as compared to virus control wells was considered the endpoint.

Data Analysis

Statistical comparison of presence of IgA and IgG in nasal secretions between rimantadine and placebo groups were performed by Fisher's exact test. Because of small number of laboratory confirmed infections, IgA (and IgG) in nasal secretions was considered positive if IgA (and IgG) was found in any sample from an individual during any day of treatment. Comparison of mean neutralization antibodies, lymphocyte blastogenesis and natural killer cell activity was made by analysis of variances. Multiple regression analysis was used to analyze the affect of various demographic and illness characteristics on the above test results.

RESULTS

Fifty families were initially enrolled prior to the influenza season. During the influenza season, 23 families consisting of 38 adults and 46 children were started on placebo or rimantadine as described previously [Hayden et al., 1989]. Blood samples for analysis were obtained from 32 of 44 ill family members and from 9 non-ill adults. Failure of obtaining specimens was either due to the individual not being available for blood drawing when the nurse made the home visit or the patient refused to have blood drawn. Inadequate samples were obtained from three individuals in both the placebo and rimantadine groups to allow lymphocyte blastogenesis assays to be performed.

Virus Isolation and Laboratory Documented Infection

Influenza A (H3N2) was isolated from nasal secretions of 7 of 21 ill adults and 6 of 23 ill children. Laboratory documented infection occurred in 5 of 17 adults and 5 of 22 children in the rimantadine group and 4 of 21 adults and 8 of 24 children in the placebo

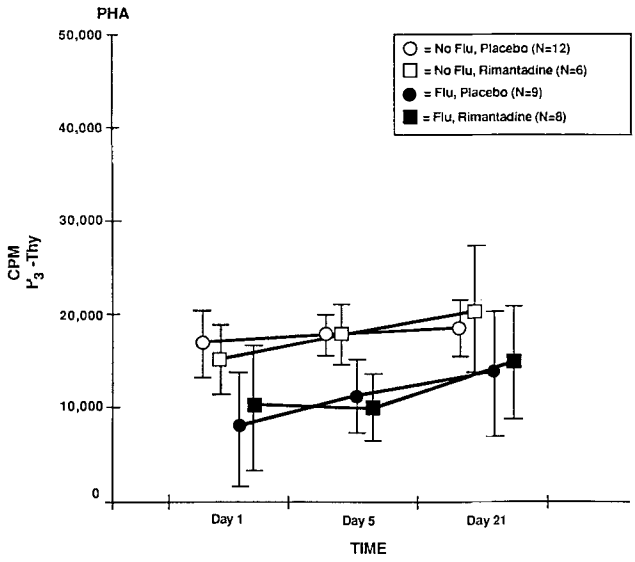

Fig. 1. Mean lymphocyte response to PHA vs. time. Error bars represent 95% confidence intervals.
Day 1—Rimantadine vs. placebo, P = NS; flu vs. no flu, $P < 0.01$.
Day 5—Rimantadine vs. placebo, P = NS; flu vs. no flu, $P < 0.02$.
Day 21—Rimantadine vs. placebo, P = NS; flu vs. no flu, P = NS.

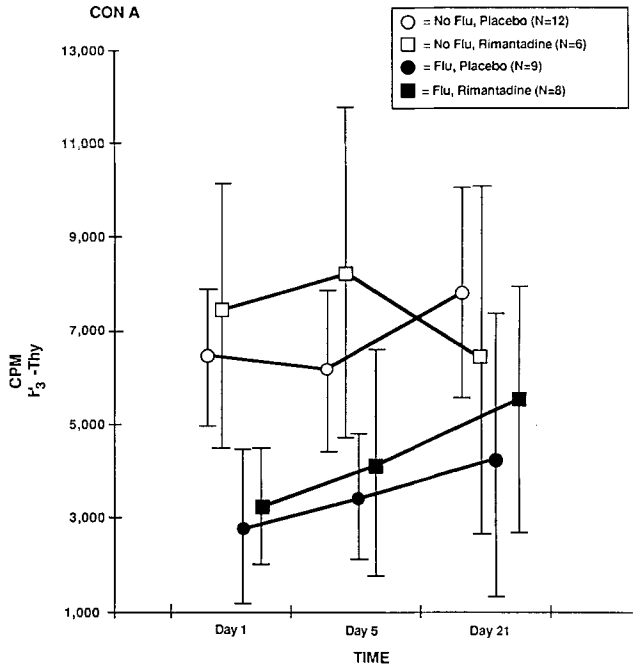

Fig. 2. Mean lymphocyte response to CON-A vs. time. Error bars represent 95% confidence intervals.
Day 1—Rimantadine vs. placebo, P = NS; flu vs. no flu, $P = 0.003$.
Day 5—Rimantadine vs. placebo, P = NS; flu vs. no flu, $P < 0.005$.
Day 21—Rimantadine vs. placebo, P = NS; flu vs. no flu, P = NS.

group. No resistant viruses were isolated, and no viruses were isolated after day 5 of illness in either group.

Mitogen Responses and Natural Killer Cell Activity

Analysis of T cell functions revealed no difference between T lymphocyte response to CON-A or PHA with respect to rimantadine versus placebo (Fig. 1 and 2). However, influenza infection itself appeared to depress T lymphocyte response to CON-A and PHA as has been previously reported by Lewis et al. [1986]. There was no significant difference in natural killer cell activity among the rimantadine and placebo groups regardless of whether they were infected with influenza A or not (Fig. 3). No significant changes occurred in these analyses when regressed on age, size of family, preexisting influenza titer, and severity of illness.

Antibody Response to Influenza A

The mean neutralizing antibody response to influenza A virus isolated proven infections was similar in the rimantadine and placebo groups (1:8 to 1:40 for rimantadine group and 1:12 to 1:72 for placebo group, P = NS). However, we detected IgA in the nasal washes of 6 out of 9 individuals with documented influenza A infections who were on placebo, compared with zero out of 9 individuals with documented influenza infections who were on rimantadine ($P < 0.005$). Moreover, IgA or IgG was detected in 7 out of 9 individuals versus zero out of 9 individuals with influenza A who were on placebo and rimantadine, respectively ($P < 0.001$).

DISCUSSION

In this study, we saw no adverse effect of rimantadine on the blastogenic response of lymphocytes. In in vitro studies [Koff et al., 1979], rimantadine decreased lymphocyte blastogenesis to Con-A at concentrations of 10 μg/ml and PHA at concentrations greater than 25 μg/ml. Concentrations less than 10 μg/ml had little or no effect on lymphocyte stimulation. In normal healthy adults, the steady state peak plasma levels of rimantadine are 0.4 to 0.5 μg/ml [Willis et al., 1987]. The fact that we did not see an adverse effect with the normal dosing of rimantadine was as expected.

Of interest, we did document the ability of the immune system to make a humoral response to influenza A infections even with treatment with rimantadine. We acknowledge that our sample size was small, and with a larger study population a diminished response may be found. Diminished antibody responses have previously been reported with other antiviral agents, specifically with acyclovir in the treatment of herpes simplex [Ashley et al., 1986; Bernstein et al., 1984; Wade et al., 1984], and with amantadine in the treatment of influenza [Reuman et al., 1989].

Of note, however, was the lack of IgA or IgG in the nasal secretions of those that were on rimantadine as compared to placebo. We hypothesize that this effect is not due to a direct effect of rimantadine on the immune system, but a result of a significant reduction by

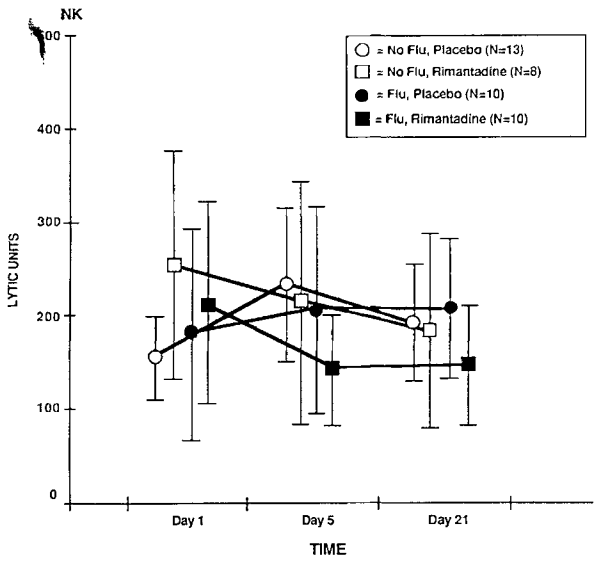

Fig. 3. Mean natural killer cell activity in lytic units vs. time. Error bars represent 95% confidence intervals.

rimantadine in the amount of influenza virus present in nasal secretions. Rimantadine is present in nasal secretions at an average of 50 percent higher than in plasma [Hayden et al., 1985; Tominack et al., 1988]. As has been previously reported [Hall et al., 1987], rimantadine does decrease the amount of viral shedding. If the amount of virus is significantly reduced in the respiratory tract, one could assume that the local immune response might also be diminished.

Although no resistant viruses were isolated in our study, the immunologic changes observed might explain how resistant viruses are selected and potentially transmitted. With the inhibition or delay in the development of IgA and/or IgG in respiratory secretions of individuals on rimantadine, the drug-resistant virus has additional time to multiply and become a larger percentage of the virions produced if not the predominant species recovered in nasal secretions. In contrast, in individuals on placebo (or not taking antivirals), the immune system makes the expected response, and the production of secretory IgA diminishes the concentration of both rimantadine-sensitive and resistant strains.

It is emphasized that these immune changes may play a role in the selection of resistant viruses and not the cause of resistant virus. The genetic basis for resistance has been well described. A single amino acid change in 1 of 4 amino acid residues in the transmembrane portion of the M2 protein (residues 27, 30, 31, or 34) results in complete resistance to amantadine and rimantadine [Hay et al., 1979; Hay et al., 1985; Belshe et al., 1988; Hay et al., 1986]. Naturally occurring resistant influenza A occurs in tissue culture-grown virus populations with a frequency of 1 in 10^3 to 1 in 10^4 [Appleyard, 1977]. Although no major epidemics of resistant viruses have been reported, potential transmission of resistant viruses within families has been described [Belshe et al.,1989; Hayden et al., 1989].

Development of resistant viruses to antiviral agents is not unique to influenza viruses. Resistant viruses have been reported with the use of acyclovir [Crumpacker et al., 1982; Wade et al., 1983] and AZT [Larder et al., 1989].

In conclusion, rimantadine has no direct adverse affect on the aspects of the systemic human immune system as measured. The diminished production of IgA in nasal secretions of individuals on rimantadine is probably related to decreased antigen in the secretions, although further studies are needed to demonstrate the factors involved. It is hypothesized that the diminished production of IgA in nasal secretions of individuals who are treated with antivirals potentially contributes to the selection of resistant viruses. Further studies are needed to study the clinical benefit of combined immunization, especially live-attenuated vaccines, chemoprophylaxis, and the development and transmission of resistant influenza viruses.

ACKNOWLEDGMENTS

We are grateful to Michael Linville for excellent technical assistance and to LaVonne Wolfe for her excellent assistance in manuscript preparation.

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