EFFECT OF INOSIPLEX ON THE HUMORAL AND CELL-MEDIATED IMMUNE RESPONSES TO INTRADERMAL HUMAN DIPLOID CELL RABIES VACCINE

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INTRODUCTION

Many intradermal (i.d.) immunization regimens of human diploid cell rabies vaccine (HDCV) have been tried, mainly to reduce the effective dose of the expensive but most widely accepted antirabies vaccine, the HDCV (Bernard *et al.*, 1982 ; Wasi *et al.*, 1983; Warrell *et al.*, 1984). We have recently studied a 4-site i.d. regimen of HDCV (0.1 ml i.d. at 4 sites on days 0, 3 and 7, followed by 0.1 ml i.d. at 1 site on day 28 and 91) and found that the antibody response was similar to the full-dose intramuscular regimen (Ubol and Phanuphak, 1986). In addition, this i.d. regimen was more effective than the intramuscular regimen in the induction of specific cell-mediated immune response (CMIR) to the vaccine (Ratanavongsiri *et al.*, 1985).

Inosiplex (Isoprinosine$^{(R)}$), a complex of inosine, N, N-dimethylamino-2-propanol and p-acetamidobenzoic acid in a 1 : 3 : 3 molar ratio, is described as an antiviral and immunopotentiating drug (Ginsberg and Glasky, 1977). It enhanced the production of specific antibody-forming cells in mice if given during the period of immunization (Renoux *et al.*, 1979). *In vitro*, the drug was found to increase the mitogen and alloantigen-stimulated lymphocyte transformation both in the mouse (Renoux *et al.*, 1979) and in the human system (Wybran *et al.*, 1978). We have shown that concurrent administration of inosiplex during the first 10 days of i.d. HDCV immunization resulted in a trend towards higher antibody levels (Ubol and Phanuphak, 1986). In this study, we further investigated whether inosiplex given with another regimen of i.d. HDCV, given at 8 sites, would enhance the antibody response. The effects of inosiplex on the T cell number and on the mitogen and antigen-stimulated transformation of lymphocytes were also investigated.

MATERIALS AND METHODS

Subjects: Thirty healthy medical and veterinarian volunteers (laboratory personnel and students) were recruited in the study. None had previously received rabies vaccine and none was taking any drugs. They were randomly allocated into 2 groups of 15 each.

Group 1 : 8-site i.d. HDCV. The subjects were given 0.1 ml HDCV (Institut Meriuex, Lyon, France, lot Y 0162 with a potency of 2.82 IU/ml as determined by NIH test) i.d. at each of eight sites (both deltoids, thighs, and both sides of anterior abdominal wall and suprascapular areas) on day 0. On day 7, they received another 4-site injection, 0.1 ml each, i.d. on both deltoids and thighs. It was found after the completion of the study that 2 subjects in this group developed an accelerated secondary-type of antibody response to the first dose of HDCV,

and they admitted to having had previous rabies vaccination. Therefore, only 13 subjects remained eligible for statistical analysis in this group. Ten were males and 3 were females with a mean age of 25.5 years.

Group 2 : i.d. HDCV + inosiplex. In addition to the 8-site i.d. HDCV as for group 1, the subjects in this group also received 50 mg/kg/day of inosiplex (Isoprinosine(R), Newport Pharmaceuticals, Newport Beach, California, U.S.A.) divided into 3-4 doses daily taken orally during the first 10 days of immunization (day 0 to day 9). Fourteen subjects in this group were males and 1 was female with a mean age of 25.3 years.

Titration of rabies neutralizing antibody

Blood samples were taken on days 0, 7 and 14. Sera were coded and sent to Dr. G.M. Baer's laboratory at the Centers for Disease Control, Atlanta, Georgia, U.S.A. for rabies neutralizing antibody titration performed blind, by the rapid immunofluorescence focus inhibition test (RIFFIT) (Smith *et al.*, 1973). The lowest detectable antibody level in the laboratory was 0.1 IU/ml, and, for computational purposes only a level of 0.01 IU/ml was assigned for results of < 0.1 IU/ml. The geometric mean titres of the groups were compared by Student's t-test.

Lymphocyte transformation test

Twelve ml of heparinized blood (10 units of heparin, Leo Pharmaceutical Products, Ballerup, Denmark, per 1 ml of blood) was obtained aseptically from each volunteer on day 0, 7, 14 and 28 and the specimen were tested blind. Mononuclear cells were separated by Ficoll-Hypaque density gradient (Boyum, 1968) using 10 parts of sterile Hypaque Sodium 33% (Winthrop Laboratories, Division of Sterling Drug Inc., N.Y., U.S.A.) mixed with 24 parts of sterile 9% Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Uppsala, Sweden). A 1 : 5 dilution of HDCV (Institut Merieux, Lyon, France) was used as the stimulating antigen and phytohaemagglutinin (PHA-P, Difco, Detroit, U.S.A.) at a final concentration of 1 μg/ml was used as the mitogen in the culture system. The triplicate cultures, containing 100 μl of mononuclear cells (2×10^6 cells/ml) and 100 μl of antigen or mitogen, were performed in flat-bottomed microculture plates (Sterilin, Teddington, Middlesex, England) as previously described (Ratanavongsiri *et al.*, 1985). The proliferative response was assessed by the amount of the tritiated thymidine incorporated during the last 16 hour pulse for the 5-day antigen culture or during the last 6 hour pulse for the 3-day mitogen culture system. The lymphocyte reactivity was expressed as Δ CPM (the difference between stimulated and unstimulated cultures) and as stimulation index (S.I., i.e., the ratio between stimulated and unstimulated cultures). The difference within the group and between the groups was tested by Student's t-test.

E-rosette forming cells

A portion of the mononuclear cells as obtained above was saved for enumeration of T cells by an overnight E-rosette forming technique (Mendes *et al.* 1973). Mononuclear cells with three or more adherent sheep red blood cells were counted as erythrocyte-rosette-forming cells (E-RFC). The number of rosetting cells was expressed as a percentage of a total of 200 mononuclear cells.

RESULTS

Rabies neutralizing antibody

None of the 28 subjects had detectable rabies antibody before receiving HDCV (i.e., day 0). Following i.d. immunization with HDCV, the antirabies antibodies rose progressively as shown in Fig. 1. On day 7, 3 out of the 13 subjects in group 1 and 1 out of

the 15 in group 2 developed detectable rabies antibodies but all were less than the hypothetical protective level of 0.5 IU/ml. By day 14, all subjects had antibodies above 0.5 IU/ml. The GMT of group 1 was 8.48 IU/ml (range : 2.0-40.0) and that of group 2 was 5.85 IU/ml (range : 0.5-22.9). The difference between the 2 groups was not statistically different.

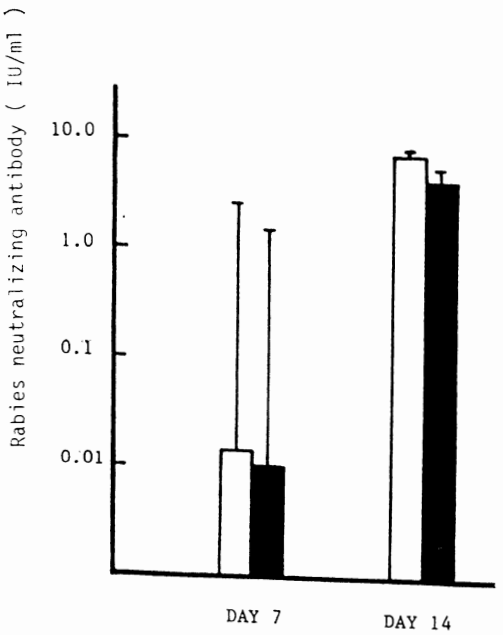

Fig. 1—Geometric mean titre of rabies neutralizing antibodies in recipients of intradermal HDCV (white column) and intradermal HDCV plus inosiplex (dark column). Bars indicate the standard error of the mean.

Antigen-stimulated lymphocyte proliferative response

Antigen-stimulated lymphocyte transformation became evident in both groups of vaccinees as soon as 7 days after starting immunization (Table 1). The response was HDCV-dependent since there was no response before HDCV immunization (day 0). The stimulation reached a maximum on day 14, had declined by day 28. No significant difference was noted between the 2 groups. No statistical correlation was found between the antibody titres and the HDCV-induced lymphocyte proliferative response ($r = 0.27$).

Effect of inosiplex on T cells and the mitogen response

In order to evaluate the immunopotentiating effect of inosiplex, the percentage of T cells and the T cell mitogen (PHA) - induced proliferative response were compared in the 2

Table 1

Antigen-stimulated lymphocyte transformation in recipients of intradermal HDCV.

Day	Group I : i.d. HDCV (N = 13)		Group II : i.d. HDCV + Inosiplex (N = 15)	
	Δ cpm*	S.I.**	Δ cpm	S.I.
0	135 ± 270***	2.0 ± 0.4	−65 ± 227	1.4 ± 0.3
7	3,947 ± 2,141	4.7 ± 1.5	3,000 ± 971	5.8 ± 1.6
14	13,523 ± 3,393	21.6 ± 6.5	14,141 ± 3,472	19.4 ± 5.9
28	6,001 ± 1,998	14.6 ± 6.2	5,290 ± 1,241	10.1 ± 2.0

* stimulated cpm - unstimulated cpm.
** stimulated cpm/unstimulated cpm
*** mean ± SEM.
Statistics: All the values on days 7, 14 and 28, either in group I or group II were significantly higher ($p < 0.05$) than the values on day 0 of the same group; but no significant difference between the groups.

Table 2

Effect of inosiplex on the percentage of T cells and on the phytohemagglutinin-induced lymphocyte transformation.

Day	Group I i.d. HDCV (N = 13)		Group II i.d. HDCV + Inosiplex (N = 15)	
	PHA (Δ cpm)	%T	PHA (Δ cpm)	% T
0	82,679 ± 12,846**	66 ± 2	78,068 ± 10,760	67 ± 2
7	59,484 ± 6,491	68 ± 2	68,416 ± 6,337	69 ± 2
14	75,171 ± 7,521	71 ± 2	60,696 ± 5,589	68 ± 2
28	59,383 ± 6,788	66 ± 3	60,559 ± 5,896	67 ± 1

* stimulated cpm - unstimulated cpm.
** mean ± SEM.

groups of i.d. HDCV recipients. There was no significant difference in either of these indices of T cell parameters whether or not inosiplex was given during the first 10 days of immunization (Table 2). The results also indicate that i.d. immunization with HDCV had no effect on the T cell numbers or on the proliferative response of these lymphocytes to the T cell mitogen, when these parameters were followed for as long as 28 days.

Side effects of treatment

As expected, symptoms associated with vaccination included itching at the injection site in all but 4 patients; feverish feelings, headache and flu-like symptoms in a few. Most subjects had some local erythema and induration for a few days following injection and half had regional lymphadenopathy. The incidence of these side effects was the same in the two groups. Nausea was reported by 3 patients taking inosiplex, two of whom also vomited once which did not interfere with the continued ingestion of the drug. One subject not taking the drug also had nausea.

DISCUSSION

Eight-site intradermal (i.d.) immunization with HDCV resulted in rapid induction of specific CMIR within 7 days. The lymphocyte reactivity reached a maximum by day 14. This confirms our previous findings that specific CMIR can be readily and consistently induced by the multisite i.d. HDCV immunization (Ratanavongsiri *et al.*, 1985). In our earlier study, a 4-site i.d. regimen induced a CMIR about 7 days earlier than the full-dose intramuscular (i.m.) regimen. Such rapid induction of CMIR may be advantageous in rabies prophylaxis. This is of particular interest since rabies encephalitis may develop in individuals with readily demonstrable neutralizing antibodies (Devriendt *et al.*, 1982). We found no statistical correlation between the levels of neutralizing antibody and the degree of lymphocyte reactivity, which agrees with a previous study (Ratanavongsiri *et al.*, 1985). The relative importance of the different effector arms of the immune response in protection against rabies has yet to be established.

Inosiplex has been tried in several controlled studies to treat viral infections such as rhinovirus, influenza virus, measles and herpes virus with variable results (Waldman and Ganguly, 1977; Longley *et al.*, 1973; Corey *et al.*, 1979; Feldman *et al.*, 1978; Haddad and Risk, 1980). However, its *in vitro* antiviral effect is relatively weak compared to

other antiviral drugs (Ohnishi *et al.*, 1983). As a consequence, its *in vivo* antiviral effect is thought to be the result of the drug's immunopotentiating effects on other inflammatory and immune cells such as neutrophils, monocytes, macrophages as well as T and B lymphocytes (Ginsberg and Glasky, 1977; Wybran *et al.*, 1978 ; Chang and Heel, 1981). Administration of inosiplex to patients with various viral infections restored the functions of T cells which were suppressed by the viruses (Waldman and Ganguly, 1977; Corey *et al.*, 1979). On the other hand, the *in vivo* effects of inosiplex on the immune functions on the normal or immunocompetent individuals, such as the volunteer vaccinees, have rarely been studied.

Unlike previous findings (Ubol and Phanuphak, 1986), there was no trend towards an increased antibody response in subjects treated with inosiplex. Different immunization schedules were used in these 2 studies. In the first one, a 4-site i.d. regimen split the 8-site injection on day 0 into 2, of 4 sites each on days 0 and 3. The immunopotentiating effect of inosiplex may depend on the antigen load and the frequency of immunization.

The lack of *in vivo* effect of inosiplex on the total T cell number and PHA-induced blastogenesis found in our studies is consistent with other previous reports in man (Galbraith *et al.*, 1984). However, certain subsets of lymphocytes such as the auto-rosette forming cells which possessed the phenotypic markers of helper T cells, were found to increase after *in vitro* incubation with inosiplex (Rey *et al.*, 1983). Similarly, PHA-induced blastogenesis of normal peripheral blood mononuclear cells could be enhanced by *in vitro* incubation with inosiplex (Nakamura *et al.*, 1983; Hadden *et al.*, 1976). The discordance between the *in vivo* and the *in vitro* effects of inosiplex has also been exemplified by the studies of Hersey *et al.* that suppressor T cell functions could be enhanced by *in vivo* administration of inosiplex whereas *in vitro* incubation resulted in decreased suppressor T cell functions (Hersey *et al.*, 1984). The apparent effect of inosiplex on the immune response may therefore be affected by the immunocompetence of the individual and the nature of the test used to monitor the response.

SUMMARY

Antigen-stimulated lymphocyte transformation was studied in recipients of intradermal human diploid cell rabies vaccine (HDCV). HDCV was administered intradermally at 8 different anatomical sites, 0.1 ml each, on day 0; followed by another 4-site injection on day 7. Rabies antigen-stimulated *in vitro* proliferative response was evident as early as 7 days after starting immunization. It reached a peak on day 14 and had declined by day 28. The cellular proliferative response preceded and roughly correlated with the antirabies antibody response. Simultaneous administration of inosiplex, an antiviral and immunopotentiating drug, during the first 10 days of intradermal HDCV immunization did not result in heightened antibody titres or cell-mediated immune response to the vaccine. The number of T cells and the lymphocyte proliferative response to phytohaemagglutinin in inosiplex-treated vaccinees were similarly not significantly different from untreated controls. Our results confirm other previous findings that a specific cell-mediated immune response can be consistently and rapidly induced by an intradermal regimen of HDCV immunization. The addition of inosiplex to this regimen did not enhance the humoral or cell-mediated immune responses to the vaccine. The apparent lack of immunostimulating effect of inosiplex in this setting may be the result of several factors such as the immunization schedule and the immunologic parameters examined.

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