Cell-mediated Immune Response Following Intracutaneous Immunisation with Human Diploid Cell Rabies Vaccine*

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SUMMARY Specific cell-mediated immune response (CMIR) against rabies antigens was studied in recipients of two regimens of human diploid cell rabies vaccine (HDCV) using the antigen-stimulated lymphocyte transformation test (LTT) as a measure of CMIR. Reconstituted HDCV could be conveniently used as the *in vitro* stimulating antigen and the response was antigen-dependent. Conventional intramuscular immunization with full-dose HDCV resulted in positive LTT as early as 14 days after starting immunisation, and peaked on day 28. Intracutaneous immunisation with 0.1 ml of HDCV at four sites on days 0, 3 and 7 was a more efficient means of inducing specific lymphocyte response. Specific CMIR was evident as early as seven days and became maximal on day 14. In addition to the more rapid induction of specific CMIR, our intracutaneous regimen also resulted in a brisker and higher antibody response than the intramuscular regimen. The peak antibody level of the intracutaneous regimen was reached on day 14 whereas that of the intramuscular regimen was reached on day 28 and the geometric mean antibody titre on day 14 of the intracutaneous route was significantly higher than that of the intramuscular regimen. We therefore conclude that our closely spaced intracutaneous immunisation with HDCV was effective both in the induction of specific antibodies and the cell-mediated immune response.

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Rabies is a major public health problem in many countries and post-exposure vaccination is a standard measure for preventing the disease. The level of rabies neutralising antibody is generally accepted as an index of effective immunisation and, although the protective level of the antibody titre has yet to be established, WHO has recommended that vaccination regimens should produce rabies neutralising antibody in excess of 0.5 I.U./ml.[1] However, instances of human rabies have been reported in individuals with readily demonstrable neutralising antibody[2-5] thus raising questions about its protective role. In consequence we have investigated another effector arm of the immune response, the cell-mediated immune response (CMIR) following rabies vaccination.

In this study, we examined CMIR against rabies virus following the administration of human diploid cell rabies vaccine (HDCV) in man by the method of antigen-stimulated lymphocyte transformation test. The results of the new, economical, closely spaced, multisite, intracutaneous regimen were compared with those using the conventional full-dose intramuscular regimen.

MATERIALS AND METHODS

Subjects

Sixteen patients with low-risk exposure to rabies, attending the Rabies Clinic at Queen Saovabha Memorial Institute, were included in the study. None had been previously immunised with rabies vaccine. They were divided into two groups. The first group, consisting of five males and five females ranging in age from 14 to 54 years ($\bar{x}$ ± SD = 33.3 ± 12.3), received four

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intracutaneous injections of 0.1 ml of human diploid cell rabies vaccine (HDCV: Institut Merieux, Lyon, France) each, distributed over both thighs and arms on days 0, 3 and 7. This was followed by another 0.1 ml of HDCV injected intracutaneously in the deltoid area on days 28 and 91. The second group, two males and four females, ranging in age from 13 to 62 years ($\bar{x} \pm SD$ = 26.9 ± 13.8) received 1.0 ml of HDCV intramuscularly on days 0, 3, 7, 14, 28 and 91.

Lymphocyte transformation test

Twelve millilitres of heparinised blood (10 units of heparin (Leo Pharmaceutical Products, Ballerup, Denmark) per 1 ml of blood) was obtained aseptically from each patient on days 0, 7, 14, 28 and 91. Mononuclear cells were separated from the heparinised blood using Ficoll-Hypaque density gradient; 10 parts of sterile hypaque sodium 33% (Winthrop Laboratories, Division of Sterling Drug Inc., NY, NY, U.S.A.) were mixed with 24 parts of sterile 9% Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Uppsala, Sweden). One hundred microlitres of mononuclear cell suspension (2 x 10^6 cells/ml) in a culture medium containing RPMI 1640 (Grand Island Biological Company, Grand Island, NY, U.S.A.), 10 mM HEPES (also from Grand Island Biological Company), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO, U.S.A.), 100 units/ml of penicillin G, 100 μg/ml of streptomycin and 5% human AB serum, were cultured in flat-bottomed microculture plates (Flow Lab, Dubrin, VA, U.S.A.) with 100 μl of 1:5 HDCV (Institut Merieux, Lyon, France), as antigen. The cultures were performed in triplicate and were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for six days. Eighteen hours before harvest, 20 μl of 0.5 μ Ci ^{3}H-thymidine (specific activity 8.3 mCi/mg: Amersham, Buckinghamshire, U.K.) were added; ^{3}H-thymidine incorporation was determined by harvesting cells with a multichannel automatic cell harvester (CH-103, Dynatech Lab, Inc., Sussex, U.K.) and the radioactivity was measured in a liquid scintillation counter.

The lymphocyte reactivity was expressed as Δ CPM, i.e., the difference between sitmulated and unstimulated cultures. The siginificance of the difference between the groups and within each group was tested by the Student's t-test.

Neutralising antibody titration

Serum neutralising antibodies to rabies virus were assayed on days 0, 7, 14, 28 and 91 by the rapid immunofluorescent focus inhibition test (RIFFIT) according to the method of Smith *et al.*[6] All serum specimens were heat-inactivated at 56°C for 30 minutes before assay. The international standard antiserum to rabies virus was a gift from Dr. Urich Bijok, Behring Institute, West Germany and was included in each assay. The antibody levels were expressed as international units per millilitre and the geometric mean titres (GMT) were calculated for each group. The Student's t-test was used to calculate the significance of the difference between the two GMTs. For computational purposes, the value of 0.1 IU/ml was assigned to the undetectable titre since the lowest detectable antibody level in our assay system was 0.2 IU/ml.

RESULTS

Lymphocyte transformation test

In the intracutaneous (I.C.) HDCV group, significant antigen-stimulated lymphocyte transformation was evident within seven days of starting the immunisation regimen (Fig. 1). Seven of the 10 subjects in this group had a stimulation index of over 2 on day 7. The lymphocyte response reached a peak on day 14, decreased slightly on day 28 and disappeared on day

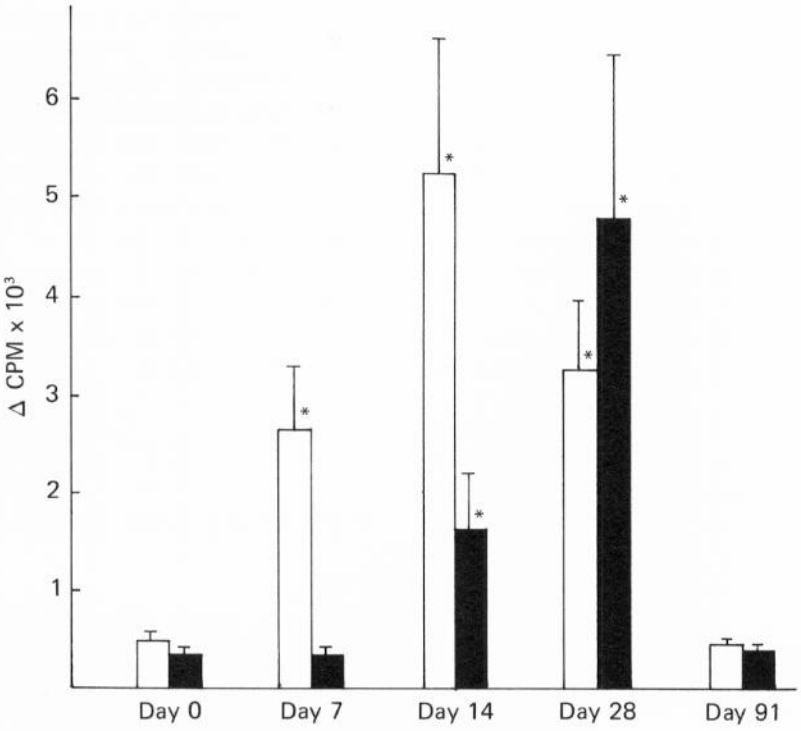

Fig. 1 Kinetics of the specific lymphocyte transformation in recipients of intracutaneous (open column) and intramuscular (closed column) HDCV. Each column represents the mean Δ cpm (stimulated cpm− unstimulated cpm) with bar as SEM. *P value < 0.05 as compared with day 0 of the same group.

91 (Fig. 1). Similar patterns of lymphocyte response were obtained when either a 1:5 or 1:25 dilution of HDCV was used as the *in vitro* stimulating antigen (data not shown) although a slightly lower response was obtained with the latter dilution.

In contrast, none of the recipients of the intramuscular (I.M.) HDCV had any antigen-stimulated lymphocyte proliferation by day 7. However, significant proliferation was seen on day 14, reaching a peak on day 28 and again disappearing on day 91 (Fig. 1). The antigen-specific lymphocyte response in the I.M. group was significantly lower than that of the I.C. group both on day 7 and day 14.

Rabies neutralising antibodies

None of the patients had detectable rabies antibodies prior to immunisation (day 0). Seven days after starting immunisation, seven of the 10 recipients of intracutaneous HDCV developed detectable neutralising antibodies, six of which exceeded the level of 0.5 IU/ml. On the contrary, only one of the six recipients of intramuscular HDCV had antibodies on day 7 which also exceeded 0.5 IU/ml. The peak antibody level of the intracutaneous group was reached on day 14, with a GMT of 12.01 ± 1.30 IU/ml whereas that of the intramuscular group was reached on day 28, with a GMT of 6.78 ± 1.57 IU/ml (Table 1). Although the antibody levels in the intracutaneous group appeared higher than those of the intramuscular regimen from day 7 through day 28, only the difference on day 14 was statistically significant ($p < 0.05$) (Table 1).

DISCUSSION

Several studies have demonstrated the reactivity of lymphocytes after immunisation with live or attenuated rabies virus in experimental animals thus suggesting the role of cell-mediated immune response (CMIR) both in the protection against and in the immunopathogenesis of rabies.[7-10] However, very little is known about CMIR in humans with rabies or following rabies immunisation.

These investigations were designed to study and compare the specific CMIR in different immunisation regimens of HDCV which are currently under trial at our Institute. We demonstrated specific lymphocyte transformation with all the immunisation regimens studied. The response was antigen-dependent since no proliferation was seen when nonsensitised lymphocytes (day 0) were tested. Reconstituted HDCV was used directly as the *in vitro* antigen since removal of aluminium hydroxide by centrifugation or of phenol by dialysis made no difference to our test system (results not shown).

Table 1 Levels of neutralising antibody in recipients of intracutaneous and intramuscular HDCV

	Intracutaneous HDCV (n = 10)	Intramuscular HDCV (n = 6)
Day 0	Undetectable	Undetectable
Day 7	0.23 ± 2.00*	0.02 ± 2.01 (N.S.)
Day 14	12.01 ± 1.30	5.26 ± 1.30†
Day 28	9.96 ± 1.24	6.78 ± 1.57 (N.S.)
Day 91	3.00 ± 1.25	3.75 ± 2.00 (N.S.)

*GMT ± SEM (IU/ml)
†P-value < 0.05 as compared with the intracutaneous group.
N.S. = No significant difference between the groups.

The lymphocyte response observed in the recipients of intramuscular HDCV confirmed the studies of Nicholson *et al.*[11] However, our studies are the first to investigate the specific CMIR in multi-site intracutaneous immunisation. Our intensive intracutaneous immunisation resulted in more rapid stimulation of CMIR than the conventional intramuscular regimen. It also resulted in a more rapid and higher antibody response. The efficacy of multi-site intracutaneous immunisation with HDCV confirms other previous studies[12,13] although slightly different immunisation regimens were used. This is particularly of economic interest since only one fourth of the HDCV used in the intramuscular regimen is needed for the intracutaneous route, also the side-effects of the intracutaneous regimen were minimal and tolerable.

There are several reasons why intracutaneous injection may produce a better immune response: 1) Intracutaneous deposition of antigen has a depot or adjuvant-like effect, resulting in slower diffusion of antigen; 2) It attracts more antigen-presenting cells since the skin is rich in Langerhans cells, the antigen-presenting cells of the skin;[14,15] 3) The rich lymphatic supply of the skin results in a more effective antigenic presentation and stimulation of the immune system. Furthermore, multi-site immunisation should ensure equal but maximal distribution of the antigens to the anatomically available draining lymph nodes. Also intracutaneous immunisation may more effectively generate helper T cells as well as effector T cells in delayed type hypersensitivity (DTH). Indeed, intracutaneous injection of soluble proteins was employed by Crowle *et al* to induce DTH in mice.[16]

Our studies demonstrated a good correlation between the humoral and cell-mediated immune responses following rabies immunisation. Nevertheless, the specific cell-mediated response disappeared faster than the antibody response,

i.e., by day 91. Which effector arm of the immune response, humoral or cell-mediated, plays the greater role in protection against rabies needs further investigation. The significance of these aspects must be evaluated in view of reports of rabies developing in persons who had good levels of neutralising antibodies.[2-5]

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