# Comparison of Human Immune Responses to Purified Vero Cell and Human Diploid Cell Rabies Vaccines by Using Two Different Antibody Titration Methods

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Antibody responses to a conventional rabies preexposure regimen of a new purified Vero cell rabies vaccine (PVRV) and a human diploid cell vaccine (HDCV) were compared in 80 healthy Kenyan veterinary students. Forty-three of the students received the PVRV and 37 received the HDCV on days 0, 7, and 28. Antibody responses were monitored by using the rapid fluorescent-focus inhibition test (RFFIT) and an inhibition enzyme immunoassay (INH EIA) on days 0, 7, 28, and 49. Both vaccines elicited a rapid antibody response. A good correlation between the RFFIT titers and the INH EIA titers was obtained ($r = 0.90$). Our results also showed that the INH EIA was more reproducible and might therefore be a suitable substitute for the more expensive and less reproducible RFFIT. The geometric mean titers determined by both tests in the two groups of students were statistically similar during the test period. The RFFIT and the INH EIA gave comparable geometric mean titers, which differed significantly only on day 28 in the PVRV group. The effect of the new PVRV is comparable to that of the more expensive HDCV, as determined by the present test systems. The PVRV could therefore be the vaccine of choice, especially in tropical rabies-endemic areas, where the high cost of the HDCV has confined its use to a privileged few.

The human diploid cell vaccine (HDCV) has been shown to be highly effective in both pre- and postexposure rabies immunization (5, 20). However, this vaccine is relatively expensive and represents a financial burden on developing nations, which suffer financial constraints as well as a high incidence of rabies. To conserve funds and vaccine, some governments have adopted regimens in which smaller doses of vaccine are given intradermally rather than intramuscularly as recommended by the manufacturer. Investigations carried out in the United States and elsewhere have indicated that in the United States and Europe, intradermal inoculation produces an adequate antibody response (6). Nevertheless, Europeans immunized by this method in a number of tropical areas develop a much poorer response (6). The response to preexposure intradermal immunization with the HDCV has been shown to be significantly lower in Kenyan citizens than in people immunized in the United States (4).

It is therefore important to assess the efficacy of other vaccines which can be made available at a lower cost. A new rabies vaccine in which Vero cells are used to produce the immunogen has been developed (13). This vaccine has been shown in a limited study in Europe to elicit antibody responses as high as or higher than those elicited by the HDCV when administered intramuscularly at an equal antigenic potency in one-half the volume used for the HDCV (13). The cost of the purified Vero cell rabies vaccine (PVRV) is approximately one-half that of the HDCV.

The mouse neutralization test and the rapid fluorescent-focus inhibition test (RFFIT) (14) have been recognized as

suitable methods for measuring the protective potency of an antirabies serum (1, 11). Both tests, however, demand highly skilled manpower. The absolute requirement for a live infective challenge virus, a large number of animals or tissue culture capability, and special facilities and equipment render these two neutralization tests unsuitable for routine or large-scale use in many countries.

In the present study, antibody titers elicited by the PVRV and the HDCV in two groups of students were studied by using both the RFFIT and a newly developed inhibition enzyme immunoassay (INH EIA). This report presents a comparison of the efficacy of the two vaccines as well as an evaluation of the new assay for the quantitation of antibodies raised against rabies virus by vaccination.

## MATERIALS AND METHODS

**Rabies vaccinees.** Eighty healthy veterinary students from the University of Nairobi, with no previous history of rabies vaccination or exposure, were recruited for the study. Forty-three of the students (34 males and 9 females) received the PVRV (Institut Merieux, Lyon, France), while the remaining 37 (35 males and 2 females) received the HDCV (Institut Merieux), in both cases by deep intramuscular injections in the gluteal region with vaccines of equal antigenic potency of 2.5 IU per dose on days 0, 7, and 28, as recommended by the manufacturers. Serial blood samples of 4 to 5 ml each were drawn prior to each injection.

**Antibody assays.** Serum samples were tested in a double-blind fashion both at the Pasteur Institute, Paris, France, by using the RFFIT (14) and at the Department of Public Health, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi, Nairobi, Kenya, by using the INH EIA, generally in accordance with the prin-

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ciples of W. Duermeyer (Ph.D. thesis, University of Amsterdam, Amsterdam, The Netherlands, 1980). The procedure used is described in detail below.

**Conjugate preparation.** An antirabies serum was produced in a goat by repeated intramuscular injections with the HDCV, to which Freund complete and incomplete adjuvants (Difco Laboratories Detroit, Mich.) had been admixed in a ratio of 1:3; Freund complete adjuvant was used only on day 1, and Freund incomplete adjuvant was used for the subsequent injections. The goat antirabies serum reacted with human serum in an Ouchterlony double-diffusion test (12) and was therefore absorbed with nonsolubilized human serum as described by Avrameas and Ternynck (2). The precipitation lines against human serum disappeared, and the absorbed serum showed only two precipitation lines against the rabies virus preparation. The immunoglobulin G fraction was isolated by the method of Fey et al. (8) with Cellex D DEAE-cellulose (Bio-Rad Laboratories, Richmond, Calif.). The immunoglobulin G fraction was concentrated by using a Diaflo PM30 ultrafilter (Amicon Corp., Lexington, Mass.) and coupled to horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) by using the heterobifunctional reagent $N$-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia, Uppsala, Sweden) by the method of Ishikawa et al. (10). The enzyme-antibody conjugate reacted with a rabies virus preparation (described below) and with the HDCV adsorbed to microtiter plates. In the INH EIA (described below), only serum samples known to contain rabies antibodies showed inhibitory activity.

**INH EIA.** Standardization of the antigen and conjugate working dilutions was established by checkerboard titrations. Microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl per well of a 1:200 dilution of an inactivated and concentrated rabies virus preparation (Flury LEP strain grown in a baby hamster kidney cell culture with an antigenicity value of 25 IU/ml; kindly donated by A. Boge, Impfstoffwerk Wellcome GmbH, Burgwedel, Federal Republic of Germany). The coating buffer was a 1:100 dilution of phosphate-buffered saline containing 2% polyethylene glycol 6000 and 0.1% sodium azide. The coated plates were incubated at room temperature in a humid chamber overnight. Plates not used the next day were stored frozen at −20°C. Before use, the plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20; test serum samples diluted in 50 mM phosphate buffer with 1 M KCl–0.3 mM disodium EDTA–polyethylene glycol 3 mM 6000–0.1% Tween 80–0.25% benzoic acid (pH 7.5) in volumes of 100 μl were added to the wells. The plates were incubated at room temperature in a humid chamber overnight and washed three times. After 100 μl of the goat antirabies immunoglobulin G-horseradish peroxidase conjugate had been added per well, the plates were incubated at 37°C for 1 h. The composition of the conjugate diluent was similar to that of the serum diluent except that it contained 0.5% Tween 80 but no polyethylene glycol 6000. After the final washing, the substrate solution was added, and the plates were incubated for 1 h at room temperature. The substrate solution consisted of 1 mg of $o$-phenylenediamine (Sigma) per ml of 0.05 M citrate-ammonium acetate buffer (pH 5.0) containing 0.1% hydrogen peroxide. The optical density of each well was read with a Minireader MR 590 (Dynatech Instruments, Inc., Santa Monica, Calif.) equipped with a 410-nm interference filter. The titer was expressed as the reciprocal of the serum dilution which gave 50% inhibition.

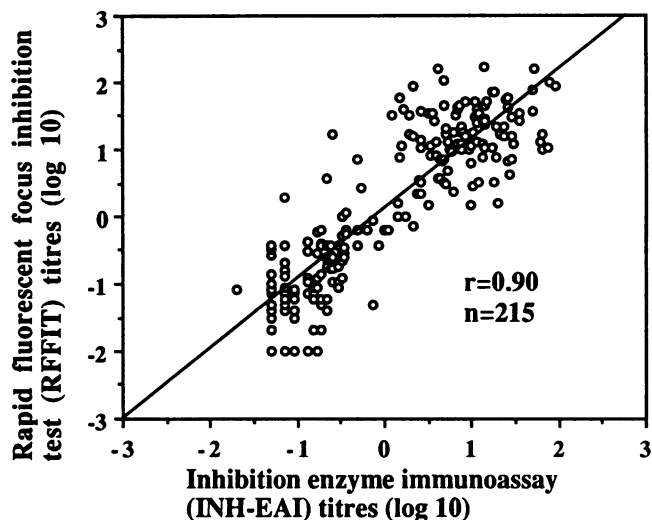**Reference sera.** The reference serum obtained from the



FIG. 1. Comparison of the RFFIT and the INH EIA with serum samples from vaccinees receiving either the PVRV or the HDCV.

Pasteur Institute (reference serum A) had a potency of 10 IU/ml and was used in the RFFIT, while the reference serum obtained from Institut Merieux (reference serum B), with a stated potency of 189 IU/ml, was used in the INH EIA. A standard human antirabies serum (reference serum C) (Imogam Rage, Institut Merieux) with a potency of 194 IU/ml was titrated against reference serum C, which had a potency of 189 IU/ml, and the results were used to determine the reproducibility of the INH EIA.

## RESULTS

**Comparison of the RFFIT and the INH EIA.** Reference serum B had a mean potency of 180.4 IU/ml (range, 167 to 195 IU/ml) in the INH EIA when tested in duplicate on five different days and calculated on the basis of standard antirabies serum C with a potency of 194 IU/ml. The coefficient of variation of the titers obtained by the test was ±4.5%. In the RFFIT, reference serum B had a potency of 189 IU/ml (range, 122 to 256 IU/ml), the coefficient of variation being ±30%. A correlation coefficient of $r = 0.90$ was obtained between the results obtained from the RFFIT and those obtained from the INH EIA for all of the serum samples from all of the students receiving the vaccines (Fig. 1). The only significant difference ($P = 0.002$) between the two tests concerned the results obtained for serum collected on day 28 in the group receiving the PVRV.

**Comparison of the immune responses elicited by the two vaccines.** Table 1 shows the results of the vaccination experiments. Thirty-four percent of the students receiving the PVRV were considered to have seroconverted (had antibody titers equivalent to ≥0.5 IU/ml) on day 7, and all had seroconverted by day 28, as determined by the RFFIT. Only 17% of the students receiving the HDCV had seroconverted on day 7, although 100% had done so by day 28, also as measured by the RFFIT. Similar, although lower, figures were obtained when these sera were measured by the INH EIA. On day 7, the PVRV elicited a higher geometric mean titer (GMT) than did the HDCV. The difference was, however, not statistically significant for either of the test methods. Twenty-one days after the second vaccination, there was a marked increase in the GMT in the PVRV group as

TABLE 1. GMTs, ranges of GMTs, and percent seroconversion rates in the PVRV group and the HDCV group, as determined by the RFFIT and the INH EIA

| Test | Vaccine | GMT (range of GMT) [% seroconversion rate] on day: | | | |
|------|---------|---|---|---|---|
| | | 0 | 7 | 28 | 49 |
| RFFIT | PVRV | 0.11 | 1.13 (0.05–17.45) [34] | 31.52 (11.20–66.82) [100] | 32.80 (6.68–145.62) [100] |
| | HDCV | 0.16 | 0.34 (0.01–1.11) [17] | 18.28 (0.64–145.67) [100] | 25.83 (1.51–103.11) [100] |
| INH EIA | PVRV | 0.12 | 0.45 (0.05–2.19) [24] | 10.48 (1.47–49.35) [100] | 17.90 (1.91–73.31) [100] |
| | HDCV | 0.11 | 0.29 (0.05–1.11) [14] | 9.83 (0.48–52.53) [97] | 23.31 (1.23–91.02) [100] |

compared with the HDCV group. Nevertheless, there was still no statistical difference between the GMTs in the two vaccine groups, as determined by the two immunoassays; this was also the situation after 49 days. Fourteen vaccinees (7 of whom had received the PVRV and 7 of whom had received the HDCV) showed a fall in titer from days 28 to 49 when tested by the RFFIT but an increase when tested by the INH-EIA, as did all of the other 66 vaccinees. No difference in the measured immune responses between males and females was observed.

## DISCUSSION

In this comparative study, both the PVRV and the HDCV elicited an antibody response in the 80 human volunteers which could be detected as early as day 7 after a single vaccine injection. Seroconversion rates on day 7, as judged by the presence of antibody titers equivalent to $\geq 0.5$ IU/ml (the level set by the World Health Organization), were 24 and 34% for the PVRV group and 14 and 17% for the HDCV group, as determined by the INH EIA and the RFFIT, respectively. These results are in contrast to those obtained in another study in which neither the HDCV nor the PVRV induced an antibody titer equivalent to 0.5 IU/ml by day 7, even though the vaccinees had received injections on both days 0 and 3 (16). This discrepancy may to some extent have been due to the fact that only 15 vaccinees per group were used in that study. The antibody determination may also have been adversely affected by interference from any antigens circulating in the serum as a result of vaccination only 3 days previously (16).

The GMT induced in the PVRV group was slightly higher than, although not significantly different from, that induced in the HDCV group on day 7. This was true irrespective of the assay technique used. These results compare very well with those obtained in another comparative study conducted in France, in which GMTs of 1.0 and 0.5 IU/ml in the PVRV and the HDCV groups, respectively, were induced by day 7 (P. Sureau, P. E. Rollin, C. Fritzell, M. Y. Touir, and M. Lajon, 1st Int. Conf. Impact Viral Dis. Dev. Asian Countries, Bangkok, Thailand, 1986, p. 16–18).

By day 28, all vaccinees had antibody titers equivalent to or above 0.5 IU/ml. In another trial of the PVRV, in which a preexposure immunization schedule of days 0, 7, and 21 was used, a 100% seroconversion rate was demonstrated by day 35 (17). The GMT of 13.18 IU/ml (range, 1.4 to 43.0 IU/ml) obtained compares favorably with our results for the PVRV group determined by the INH EIA, in which a GMT of 10.48 IU/ml (range, 1.47 to 49.35 IU/ml) was obtained by day 28 (Table 1). The GMTs of the two groups were the same by days 28 and 49, when determined by the INH EIA, but differed, although not significantly, when determined by the RFFIT. These results are in agreement with those of earlier preliminary clinical tests of preexposure and postexposure

regimens in which antibody responses to the PVRV which were equivalent to or more rapid than those reported in earlier studies with the HDCV were demonstrated (7, 9, 17).

The results obtained in this study indicate that the three-injection schedule on days 0, 7, and 28 is adequate for preexposure treatment, since a rapid and significant increase in antibody levels with a peak GMT by day 49 was observed. Similar results have been reported in earlier studies in France, Tunisia, and Yugoslavia, in which the three-injection schedule proved more effective than the two-injection schedule on days 0 and 28 (N. Ajjan, A. Strady, and M. Lienard, 1st Int. Conf. Impact Viral Dis. Dev. Asian Countries, Bangkok, Thailand, 1986, p. 18). Our results also confirm those obtained in another study in which antibody levels elicited by the PVRV were comparable to those elicited by the HDCV (15).

The precise mechanism by which rabies postexposure vaccination protects against disease and death is yet not known. Animal experiments show that glycoprotein-induced neutralizing antibody (19), cell-mediated immunity (18), and interferon (3) are all involved. It is for these reasons that the mouse neutralization test should a priori provide the most biologically relevant assessment of antibody activity. However, the method is cumbersome, difficult to standardize, and expensive. The RFFIT appears promising because of its economical use of reagents and test samples, rapidity, and suitability for testing large numbers of samples. However, the need for a fluorescence microscope as well as special facilities for the maintenance of cell cultures and live infective challenge virus renders the RFFIT inapplicable in most developing countries. The need for an inexpensive, rapid, and reproducible test still remains.

In this study, the results obtained with the RFFIT compared well with those obtained with the INH EIA. Comparable GMTs were obtained when sera from the two groups of students were tested by both tests. However, there was a significant difference in the GMTs obtained by these tests on day 28 in the PVRV group. This difference is attributed to the unusually high titers found in some day-28 sera by the RFFIT. The same sera showed a fall in titers by day 49, as determined by the RFFIT, but a rise in titers by day 49, as determined by the INH EIA. This apparent discrepancy could have been due to the inherent variability of the RFFIT. In fact, the INH EIA was shown to be far more reproducible than was the RFFIT, the coefficients of variation being $\pm 4.5$ and $\pm 30\%$, respectively. However, a good correlation between the two tests was obtained when a correlation analysis between the RFFIT titers and the INH EIA titers was performed ($r = 0.90$).

In conclusion, our results showed that the less expensive PVRV was comparable to the HDCV with regard to the investigated aspects and could therefore represent a useful alternative vaccine, especially in rabies-endemic areas in

countries with limited resources. Because of the lack of equipment and facilities for carrying out the RFFIT in most countries, the INH EIA appears to be an attractive alternative test method comparable to the RFFIT, the INH EIA being, in our experiments, even more reproducible than the RFFIT.

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