In vivo Detection of Specific Cell-mediated Immunity in Street Rabies Virus Infection in Mice

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SUMMARY

In street rabies-infected mice, in vivo expression of delayed type hypersensitivity (DTH) measured by the footpad test was revealed by challenge with inactivated fixed rabies virus (RV). The use of BCG as an adjuvant of cell-mediated immunity (CMI) was necessary for the production of significant DTH levels. Typical DTH kinetics were obtained, with a maximum at 24 h after the challenge. DTH was also found to be at highest levels 4 days after infection with street rabies virus. DTH could also be revealed with street rabies virus in RV immunized mice. Adoptive transfer of lymphoid cells from a street rabies infected donor to normal recipient mice was performed and DTH was tested with RV. Susceptibility of DTH to immunosuppression by cyclophosphamide treatment was also assayed in street rabies virus-infected mice and in adoptively-sensitized recipient mice. These results and the relationship between DTH and CMI in rabies infection and immunization are discussed.

INTRODUCTION

Rabies virus infection as well as active immunization against rabies will elicit both humoral and cellular reactions whose protective effects are still controversial (Turner, 1973). In fact, cell-mediated immunity (CMI) has been investigated only recently either in vitro (Wiktor et al. 1974, 1977a; Nozaki & Atanasiu, 1976; Atanasiu et al. 1977a; Häfvest et al. 1977) or in vivo (Turner, 1976; Lagrange et al. 1978). Only few data are to be found in the literature on CMI in street rabies. Using in vitro tests, Wiktor et al. (1977b) observed a weak CMI response in the course of street rabies infection. These authors speculated that street rabies infection might exercise an inhibitory effect on CMI.

Recently, Lagrange et al. (1978) have shown, with the aid of in vivo methods, the occurrence of CMI to inactivated rabies antigen (RV). A delayed type hypersensitivity (DTH) reaction was evoked in mice by rabies virus antigen. This reaction was shown to be correlated with specific CMI to RV. When BCG was used as a CMI adjuvant (Mackaness et al. 1974) significantly higher levels of DTH were produced in mice as measured by the footpad test. At the same time, mice were found to show enhanced resistance to an intracerebral challenge with 70 LD₉₀ of a standard live rabies virus strain (Blancou et al. 1979). In this work, we present evidence that CMI as detected by the DTH reaction is stimulated in street rabies virus infection.
METHODS

Animals. Specific pathogen-free Swiss outbred female OF1 mice were purchased from IFFA-CREDO (Domaine des Oncins, St Germain sur l’Arbresle, France). They were used at 6 to 8 weeks of age for DTH experiments.

Street rabies virus. The virus consisted of a crude homogenate of salivary glands (rabies SGH) excised from infected foxes and kept at -70 °C (a gift from Dr L. Andral, Centre d’Etude sur la Rage de Nancy, Malzeville, France). The infectivity of the virus stock was $10^7.8 \text{ LD}_{50}/\text{ml}$ as titrated by intracerebral inoculation of 14 to 16 g Swiss mice. In a few experiments street rabies virus was inactivated by irradiation for 20 min with a u.v. germicidal lamp (58 ergs/mm²/s). Inactivation was monitored by titration of the virus in mice.

Rabies vaccine (RV). Fixed rabies virus was prepared from virus grown on bovine foetal kidney cells, inactivated by betapropiolactone treatment and purified by isopycnic banding of the virus particles as described elsewhere (Atanasiu et al. 1977b). After pooling, purified and concentrated fractions were kept at -70 °C.

Control salivary gland homogenate (SGH). A crude homogenate (a gift from Dr L. Andral) was prepared from salivary glands of uninfected foxes and stored at -70 °C.

BCG Pasteur vaccine. The BCG vaccine was used exactly as specified in an earlier paper (Lagrange et al. 1978). $2.8 \times 10^6$ viable BCG micro-organisms were injected into the left hind footpad (LHFP) 2 weeks before immunization with rabies virus was attempted.

Immunization. Normal mice or BCG pre-treated mice were injected either with street rabies virus or with RV; 0.04 ml was injected subcutaneously (s.c.) through the plantar surface of the LHFP.

Footpad test. Immunized mice were treated for DTH reactions using the footpad test as previously described (Miller et al. 1973). In brief, 0.04 ml of RV was injected into the right hind footpad (RHFP) and the increase in footpad thickness was measured at various times with a gauge caliper (Schnell taster System, Kröplin, 0.05 mm). DTH reaction was expressed as the difference in thickness between feet after and before the inoculation.

Adoptive immunization was by two methods. (a) Systemic transfer: spleen cells were obtained from mice infected with street rabies 4 days previously; 5 x 10⁷ spleen cells in 0.5 ml of Hanks’ solution were injected intravenously (i.v.) into recipient mice. These mice were challenged by a s.c. inoculation of 100 HAU of RV per mouse into the LHFP immediately after the cell transfer. (b) Local transfer: lymph node cells were transferred as described by Metaxas & Metaxas-Buehler (1955); 10⁷ lymph node cells from infected donor mice were mixed with 100 HAU of RV per mouse and inoculated into the LHFP of recipient mice. In both cases, footpad swelling was measured at 3, 8, 24, 48 and 72 h after inoculation. Control groups of mice were injected with immune donor cells alone. Positive controls consisted of mice from the immune donor group which were challenged with 100 HAU of RV.

Cyclophosphamide (CY) was purchased from Laboratoires Lucien (Colombes, France); 200 mg/kg of CY was injected intraperitoneally in 0.5 ml in saline 1 h p.i.

Statistical analysis. Five to six animals were used in each experimental group. Results are expressed as the arithmetic mean and standard error of the mean (s.e. mean) within these groups and statistical significance was determined by Student’s t test for unpaired data.
**RESULTS**

*Time course of DTH reactions in mice infected with street rabies virus*

The time course of DTH reactions was followed in mice infected 4 days previously. The challenging dose was 250 HAU of RV per mouse injected into the RHFP. Increase of footpad thickness was followed 3, 8, 24, 48 and 72 h after injection of RV. The results are summarized in Fig. 1. Typical DTH kinetics were observed after street rabies infection in the BCG pre-treated mice. The increase in footpad thickness was highly significant ($P < 0.001$) 14 h after challenge in this group of mice. Elicitation of DTH could only be detected in BCG pre-treated mice.

*Eliciting dose response in street rabies infected mice*

Groups of mice under the adjuvant effect of BCG were infected with street rabies and tested with different doses of RV in the RHFP. A linear dose relationship was observed when levels of DTH were plotted against RV doses injected (Fig. 2). Only high doses of RV above 25 HAU per mouse were able to reveal the presence of DTH above interpretable levels. In experiments using street rabies virus, thus containing heterogeneous material from SGH, individual variations were usually larger than those found in experiments using RV (Lagrange et al. 1978).

*Dose response to varying street rabies virus*

Groups of BCG-pre-treated mice were infected with varying dilutions of street rabies virus. These consisted of fivefold dilutions of rabies-SGH from street rabies virus-infected foxes, which were inoculated into the LHFP. Challenge was carried out 4 days p.i. with 25 HAU of RV per mouse. The data presented in Fig. 3 show that only undiluted rabies-SGH could sensitize mice.
Induction of DTH to street rabies virus in RV-immune mice

The ability of RV immunized mice to develop DTH to street rabies virus infection has been tested. Groups of mice pre-treated either with BCG or saline 14 days previously were immunized with 5 HAU of RV per mouse and challenged with street rabies virus 7 and 21 days later. A highly significant DTH reaction was observed in the BCG group on day 7 ($P < 0.001$, 24 h after challenge), which persisted until day 21 (Fig. 4a). In the group of mice pre-treated with saline, lower but significant DTH reaction was observed on day 7 ($P < 0.01$), but footpad swelling on day 21 was not statistically different from that in the control group (Fig. 4b). BCG and saline control mice did not show any DTH reaction to RV at any time. But with SGH from uninfected foxes in BCG and RV-immunized mice a local inflammatory reaction was observed on days 7 and 21.

Kinetics of DTH in the course of street rabies virus infection

Groups of normal or BCG pre-treated mice were injected s.c. with street rabies virus into the LHFP, and DTH reactions were looked for as a function of time in separate groups of mice. 50 HAU of RV per mouse into the RHFP were used as the challenging dose. In the group of BCG pre-treated mice, maximum DTH levels occurred on day 4 and declined thereafter (Fig. 5a). In the group of saline pre-treated mice, weaker DTH reactions were obtained. In addition, results at day 10 in both groups of mice are doubtful since most of
Fig. 4. Increase in footpad swelling of RHFP of mice was recorded 24 h after challenge with an injection of 0.04 ml of rabies-SGH (■). As indicated, DTH reaction was tested 7 or 21 days after immunization with 5 HAU of RV per mouse in the LHFP. One group received BCG prior to immunization (a), the other group received saline (b). Control mice consisted of mice which were not immunized with RV but challenged with rabies-SGH (///). Salivary gland controls consisted of mice which were not immunized with RV but received an inoculation of control-SGH (==). Results are means of five mice ± s.e. mean.

Fig. 5. Kinetics of DTH reactions to RV were recorded in BCG (▲—▲) or saline (▲...▲) pre-treated mice in response to an injection of rabies-SGH (a) or control-SGH (b), given in the LHFP. At various times after infection, separate groups of mice were given an injection of 50 HAU of RV into the opposite footpad and 24 h footpad swellings were recorded. Results are means of five mice ± s.e. mean.
Fig. 6. DTH responses of BCG pre-treated mice 4 days after inoculation with either inactivated or infectious street rabies virus, were recorded 24 h after challenge with 50 HAU of RV per mouse. Results are means of five mice ± s.e. mean.

Fig. 7. Footpad swellings were recorded in recipient mice injected i.v. with isolated spleen cells obtained from either BCG-treated (■) or saline-treated (□) and street rabies-infected mice, and injected simultaneously with the challenging antigen (100 HAU per mouse) into the LHFP. Lymph node cells from the same donor mice were mixed with RV to obtain 100 HAU per mouse and injected s.c. into the LHFP of recipient mice and DTH reaction was recorded. Values are expressed as the relative ratio between sensitized cells and non-sensitized control cells. Results are means of five mice ± s.e. mean.

The mice were paralysed or moribund and had lost weight at that time. There were no significant differences in the incubation time, characteristics and appearance of neurological symptoms and the mean survival time among the various groups of street rabies virus-infected mice. In control, BCG mice, immunized with control SGH, challenge with RV failed to reveal specific DTH at any time up to day 10 (Fig. 5b).

The lower DTH reactions obtained with street rabies virus when compared to those obtained with RV could be a consequence of the infection. In the next experiment, inactivated street rabies virus was used to test the role of infection in the DTH reaction.

**DTH response in mice immunized with inactivated street rabies virus**

DTH responses to 5 HAU of RV were compared in groups of mice tested 4 days after inoculation with either u.v.-inactivated or live street virus. The results in Fig. 6 showed that there was no significant difference between the two groups of mice.
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Fig. 8. (a) Levels at 24 h of the reaction to 50 HAU of RV injected into the RHFP in either BCG pretreated (○) or in saline-treated (□), street rabies-infected mice which received cyclophosphamide (CY) or saline (Control). CY was injected i.p. (200 mg/kg) 1 h before infection with street rabies virus. DTH reaction was tested 4 days after challenge with RV. Results are means of five mice ± s.e. mean. (b) DTH reaction at 24 h was obtained in recipient mice injected s.c. with lymph node cells mixed with 100 HAU of RV per mouse. Sensitized cells were harvested 4 days after infection with street rabies from BCG pre-treated and CY-treated donor mice.

Adoptive cell immunity

It has been suggested that sensitized cells could be produced but are unable to circulate in street rabies virus-infected mice. This hypothesis, which would explain the weaker DTH reactions in street rabies infection, was checked here by using the transfer of immune cells. Mice pre-treated with either BCG or saline were infected with street rabies virus in the LHFP. Four and 8 days later, mice from each group were sacrificed and either spleen or popliteal lymph nodes were removed. The results obtained after systemic and local transfer of spleen and lymph node cells are summarized in Fig. 7. The highest DTH values were obtained in mice after local transfer of lymph node cells taken on day 4 from BCG pre-treated donors. DTH values were lower on day 8, but surprisingly enough, saline pre-treated mice on day 8 showed a higher DTH value than on day 4. Spleen cell transfer on day 4 provoked lower DTH reaction than local transfer from the same donors into normal recipient mice.

Immunosuppression with cyclophosphamide

In order to test why there is no high DTH reaction in mice infected with street rabies virus, CY was used to prevent the action of a positive suppressive mechanism as demonstrated by Lagrange et al. (1974). Not only was DTH not potentiated by CY treatment, but in BCG and CY pre-treated mice, DTH levels on day 4 decreased significantly (< 0.001; Fig. 8a). Since it has been shown that CY treatment causes a decrease in the number of those accessory cells which are necessary for the inflammatory reaction to
develop, the lower levels of DTH might result from the absence of such cells. To test the production of specifically-sensitized lymphocytes, lymph node cells were transferred on day 4 from CY and street rabies virus inoculated mice to recipient mice. Fig. 8(b) clearly shows that transfer was positive as far as it was carried out with lymph node cells from BCG and street rabies virus-inoculated mice, but not with cells from mice pre-treated with CY.

**DISCUSSION**

Although the pathogenesis of street rabies has been extensively studied, little is known about the role of CMI. Availability of the footpad test as an *in vivo* test allowed us to determine DTH, which reflects CMI (Mackaness, 1971; Kerckhaert *et al.* 1974; Peters *et al.* 1975), in the course of street rabies infection. However, it was necessary to use BCG as a CMI adjuvant to obtain detectable levels of DTH as revealed with RV. Mice infected with street rabies developed much lower DTH levels than mice immunized with RV. In addition, DTH was at its peak 4 days after infection with street rabies as compared to 6 to 8 days after immunization with RV. The difference in the time course of sensitization might arise from a suppressive effect exercised by rabies infection from day 4 onwards. Detection of DTH in street rabies-infected mice also required the use of as much as 25 to 50 HAU of RV per mouse. In contrast, a dose of 5 HAU of RV per mouse was effective in mice immunized with 5 HAU of RV (Lagrange *et al.* 1978). The failure of street rabies to induce a high level of DTH in mice is not dependent on an inadequate amount of immunizing antigen in the rabies-SGH, since rabies-SGH was as effective as 5 HAU of RV in detecting DTH in RV immunized mice. Since these results do not seem to be related to an insufficient amount of antigen in rabies-SGH it is possible that some sort of inhibition of CMI may be operating. This is in accordance with the results of Wiktor *et al.* (1977b) who reported the inhibitory effect of street rabies of salivary gland origin in mice which failed to develop cytotoxic T cells specific for rabies virus-infected target cells. Recently we have extended the results of Wiktor *et al.* (1977b) showing that uninfected SGH could depress immune responses to rabies (H. Tsiang *et al.* unpublished data). The similarly intense responses obtained with either virulent or u.v.-inactivated street rabies virus showed that infection does not participate in CMI inhibition. It is also safe to predict that the immune suppression seen in CMI in the course of rabies virus infection is likely to contribute to the severity of the disease.

Pre-treatment with CY has been shown to depress DTH reactions to rabies in mice (Kaplan *et al.* 1975; Tsiang & Atanasiu, 1975) or to RV (Lagrange *et al.* 1978). However, immune suppression was more effective in street rabies infection which could be ascribed to the cumulative effect of both SGH and CY. Since SGH was shown not to inhibit the production of sensitized accessory cells (H. Tsiang *et al.* unpublished data), on the one hand, and CY was shown not to abolish the expression of a positive suppressive activity (Lagrange *et al.* 1974) on the other, CMI depression by SGH and CY is likely to occur through different pathways.

Transfer of spleen cells and lymph node cells from street rabies virus-infected mice to normal recipient mice gave results in accordance with those obtained in immunized donor mice. This successful transfer points to the fact that T cells have retained their capacity to mediate DTH reaction.

Although the occurrence of CMI in the course of street rabies infection has been soundly established, its significance for rabies pathogenesis is still to be elucidated. CMI to rabies is likely to play a role in the protection against infection. In fact, Miller *et al.* (1978) reported that CMI was involved in the clearance of rabies virus from the CNS. Protection against
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rabies could also be enhanced by using BCG as an adjuvant of CMI for immunization of test animals. BCG by itself was also found to exercise a protective effect against rabies infection (Blancou et al. 1979).

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