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Delayed-Type Hypersensitivity to Rabies Virus in Mice: Assay of Active or Passive Sensitization by the Footpad Test

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With a purified beta-propiolactone-inactivated rabies virus, a significant increase in footpad swelling was elicited in normal or in BCG-pretreated mice after immunization with varying doses of rabies vaccine. These footpad reactions were shown to peak at 24 h and to be associated with an infiltration of newly formed blood monocytes demonstrated by histology and [^{125}I]deoxyuridine labeling. A relationship between the lymphoproliferation and the degree of sensitization is described, and the susceptibility to cyclophosphamide treatment is also examined. Adoptive transfer of specific reactivity to normal recipient mice with immune lymphoid cells, but not with immune serum, was demonstrated, and the results represent another argument for a cell-mediated immunological mechanism.

The role of cell-mediated immunity (CMI) in rabies infection is poorly understood. Although neutralizing antibodies are produced after active immunization, their exact role in the defense mechanism against rabies virus in vivo has not been clearly demonstrated (27). Some reports have dealt with the thymus dependency of rabies vaccine (RV); when inactivated rabies virus was injected into normal or congenitally athymic mutant mice (nu/nu), no protection and no antibody response were observed in the nude mice (28). Moreover, in vitro tests were described which clearly demonstrated a T-cell component which appears after immunization of rabbits (23, 31) or mice (30). Usually, in other infectious diseases, in vitro tests for delayed-type hypersensitivity (DTH) have been correlated with an in vivo test, all being related or not to acquired specific resistance. In tuberculosis, for instance, lymphocyte transformation or macrophage migration-inhibitory factor are demonstrated in conjunction with a positive skin test reaction to tuberculin (3). This report deals with an attempt to evaluate the production of DTH in mice immunized with inactivated rabies virus. Although DTH occurs spontaneously in the course of many infections, it seldom develops in response to artificial immunization unless powerful adjuvants or live vaccines are used. Thus, BCG infection (18) has been used here as an adjuvant for specific T-cell production in response to viral antigens.

MATERIALS AND METHODS

Animals. Specific-pathogen-free Swiss outbred female OF_1 mice purchased from IFFA-CREDO (Domaine des Oncins, St. Germain sur L'Arbresle, France) or NCS female mice bred at the Pasteur Institute were used at 6 to 8 weeks of age.

RV. Beta-propiolactone-inactivated rabies virus was prepared from bovine fetal kidney cells and purified according to methods described elsewhere (1). Two batches of RV were made, and samples were adjusted to give 250 hemagglutinin units (HAU)/ml and stocked in small volumes at −70°C.

BCG preparation. The Pasteur strain of *Mycobacterium bovis* BCG was kindly donated by M. Georghiu (BCG Production Unit, Institut Pasteur). The organisms were grown as dispersed culture in Proskauer-Beck medium containing Tween 80 and glucose. After 7 days of incubation, the cultures were frozen slowly to −70°C and were stored at this temperature. Dosage was based upon viable counts performed by plating on Middlebrook 7H10 medium. BCG was always injected 14 days before specific immunization with RV.

CY. Cyclophosphamide (CY) (donated by Laboratories Lucien, Colombes, France) was dissolved in sterile saline, membrane filtered (Millipore Corp.), and injected intravenously in a dose of 200 mg/kg on the same day as immunization (12).

Immunization. Normal mice or BCG-pretreated mice were injected with the RV, in a volume of 0.4 ml, in the plantar surface of the left hind footpad (LHFP), using a 30.5-gauge needle. Optimal doses for immunization and timing for elicitation are described in the text.

DTH. (i) Footpad test. The DTH reaction was measured as described elsewhere (21). In brief, variations of the footpad thickness were measured 4 and 24 h after injection of the eliciting antigen into the right hind footpad (RHFP).

(ii) Newly formed monocytes. As described by Miller et al. (20), immune or normal mice were injected with 0.01 ml of RV into the left ear and saline into the right ear. Nine hours after antigen administration, 0.5

ml of 10^{-3} M 5-fluorodeoxyuridine was injected intraperitoneally. Thirty minutes later, an intravenous pulse of 0.5 ml containing 1 μCi of [^{125}I]deoxyuridine (UdR) was given in the tail vein; 16 h later the mice were killed, and ears were cut off at the hair line, placed in plastic tubes, and counted in a gamma spectrometer for 10 min. The results are expressed as a ratio of the radioactivity in the left ear to that in the right ear, which was designated as left/right [^{125}I]UdR uptake.

Measurement of cellular responses in popliteal lymph nodes. The extent of cell proliferation in response to injection of RV into one footpad was measured in the draining popliteal lymph node. To measure and express the relative rates of cell division in responding nodes, [^{125}I]UdR incorporation into DNA was examined. At intervals after immunization, five mice from each group received an intravenous injection of 0.5 μCi of [^{125}I]UdR (90 to 110 μCi/μg; obtained from the Radiochemical Centre, Amersham, Great Britain). Two hours later the popliteal nodes were excised, placed in individual plastic tubes, and counted individually in a gamma spectrometer for 10 min, with uptake expressed as counts per minute. With this method, the mean background value of five tubes was subtracted from each individual count. The results are expressed as the ratio of radioactivity in the left node to that in the right node, which was designated as left/right [^{125}I]UdR uptake.

Histology. Right and left feet and right and left ears of immunized or normal mice were cut off at 4 and 24 h, respectively, after the eliciting skin test, fixed in Bouin solution and embedded in paraffin; both feet were decalcified and sectioned at 2 and 4 μm. These sections were stained with hematoxylin and eosin.

Adoptive immunization. Dissociated spleen cells or lymph node cells from immune mice were prepared as described elsewhere (14). Systemic cell transfer was performed by injecting intravenously 5×10^7 spleen cells in 0.5 ml of Hanks solution into normal recipient mice. These mice were challenged with RV in one hind footpad immediately after cell transfer. Local cell transfer was performed by the method of Metaxas and Metaxas-Buehler (19). A total of 10^7 lymph node cells or 10^7 spleen cells from immune donors were mixed with 5 HAU of RV in an 0.04-ml volume and injected into the LHFP of normal recipient mice. Footpad swelling was measured thereafter. Control groups of mice were injected with antigen alone or immune cells alone. Positive controls were groups of animals immunized at the same time as the donor mice and elicited with the same dose of RV.

Serum transfer. Serum samples were obtained from BCG-treated mice or BCG-treated and rabies antigen-immunized mice which showed high levels of DTH and were injected intravenously into groups of normal recipients in a volume of 0.5 ml. These animals then were tested 1 h later with 5 HAU of RV per mouse in the LHFP, and swelling was recorded 4 and 24 h later.

Statistical analysis. Results are expressed as the arithmetic mean and standard error of the mean. Statistical significance was determined with Student's *t* test for nonpaired data.

RESULTS

In vivo lymphoproliferative response to injection of antigen. It has been shown that the induction and the magnitude of the cellular response to a specific protein antigen is related to its antigenicity and immunogenicity (9) and that the induction of CMI is associated with increased levels of lymphoproliferation in the lymph node draining the site of antigen injection (16). Thus, the lymphoproliferative response should vary with the dose of antigen injected, and the peak of the response is related to the appearance of activated circulating T cells (11). This was tested in the following experiment. Groups of 15 normal mice were inoculated in the LHFP with varying dilutions of RV (5, 0.5, and 0.05 HAU/mouse), and one group was injected with RV diluent (20% sucrose). The cell proliferation was measured 5 days later. It was found (Fig. 1) that a log linear dose relationship exists between cell proliferation and the dose of antigen injected, and a significant increase in DNA synthesis could be detected with the highest dose compared with that obtained with the diluent ($P < 0.05$). Using this proliferative index reflecting CMI injection with the highest dose of antigen, the next experiment was undertaken to follow the kinetics of the immune response to RV. The results are shown in Fig. 2. As can be

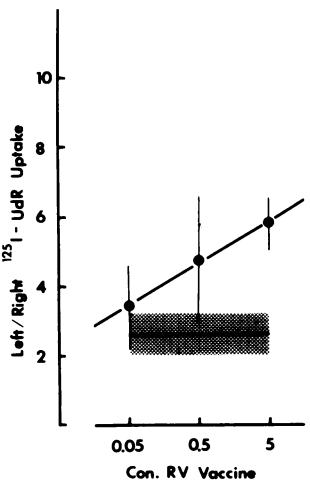

FIG. 1. *Relative rate of [^{125}I]UdR incorporation into DNA by popliteal draining lymph node cells 5 days after injection of varying doses of RV into NCS mice. Left to right: Ratios of [^{125}I]UdR uptake are computed and plotted with the dose of antigen injected. The horizontal line and area of hatching represent the mean level of the ratio (± standard error of the mean) in mice receiving only the diluent (sucrose [20%] in phosphate-buffered saline, pH 7.2). Mean of five mice ± standard error.*

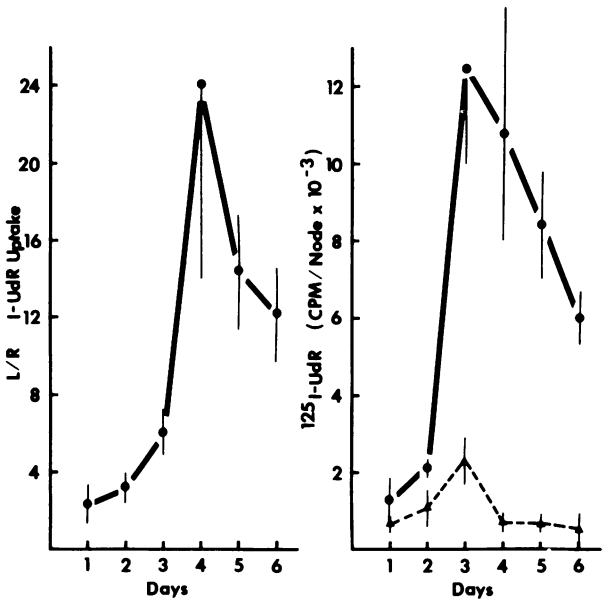

FIG. 2. *Kinetics of lymphoproliferative response in popliteal lymph nodes responding to 5 HAU of RV or the same volume of saline given at T_0 in the LHFP or RHFP, respectively, of normal OF_1 mice. (Left) Relative ratio between left lymph node and right lymph node. (Right) Absolute counts per minute per node in the left draining node (●) compared to that in the right draining node (▲). Mean of five mice ± standard error.*

seen, the peak of the immune response occurred on day 4, when the relative ratios between left draining node to right nonimmunized node were plotted (Fig. 2, left). But when absolute counts (counts per minute per node) were expressed (Fig. 2, right), the kinetics of lymphoproliferative response showed some differences. The response in the left node peaked on day 3; shortly thereafter specific proliferation diminished slowly. This disappearance was described as migration of host cells into the systemic circulation via the thoracic duct (2). Thus, these cells were tested at the periphery, and the footpad test was chosen to detect DTH to RV.

Footpad skin test. In preliminary experiments groups of 15 normal mice were injected in the LHFP with varying doses of RV, and the increase in footpad thickness was recorded 3, 24, and 48 h later. No reaction occurred at 24 h or 48 h; also, no swelling was detected with the diluent. Thus, we decided to use the highest dose of inactivated RV (5 HAU/mouse) to detect circulating effector cells after immunization in a DTH reaction. Two groups of mice were immunized with rabies antigen (5 HAU/mouse); one group was pretreated with BCG in the LHFP, and the second group received saline instead of BCG. All mice were tested 6 days after immunization with 5 HAU of RV injected into the RHFP, and the increase in footpad thickness was recorded (Fig. 3). This increase was highly significant for BCG mice at 24 and 48 h ($P < 0.001$) and even at 72 h ($P < 0.01$). A significant reaction occurred ($P < 0.005$) but to a lower degree and with a rapid disappearance, as has been described with other antigens in normal mice (11).

Eliciting dose response in immune mice. Groups of mice under the modulating effect of BCG were immunized with RV (5 HAU/mouse) into the LHFP, and 8 days later separate groups of mice were tested with different doses of RV in the RHFP. A linear dose relationship was observed when levels of DTH were plotted against the eliciting dose injected (Fig. 4), the highest levels being those in immune mice which recieved the highest dose of eliciting antigen.

Dose response curve with varying immunizing doses of antigen. To produce the highest level of DTH and also to detect possible regulatory mechanisms (17), groups of BCG-pretreated mice or groups of normal mice were immunized subcutaneously with varying doses of RV. Eight days later, all mice were tested in the RHFP with the same eliciting dose of RV (5 HAU/mouse). Results are recorded in Fig. 5. As described above, normal non-BCG-pretreated mice produced very little DTH reaction on day 8 in contrast with BCG-pretreated mice. Since it has been shown recently by Minden et al. (22) that shared antigens between melanoma cells and BCG exist and could be involved in the

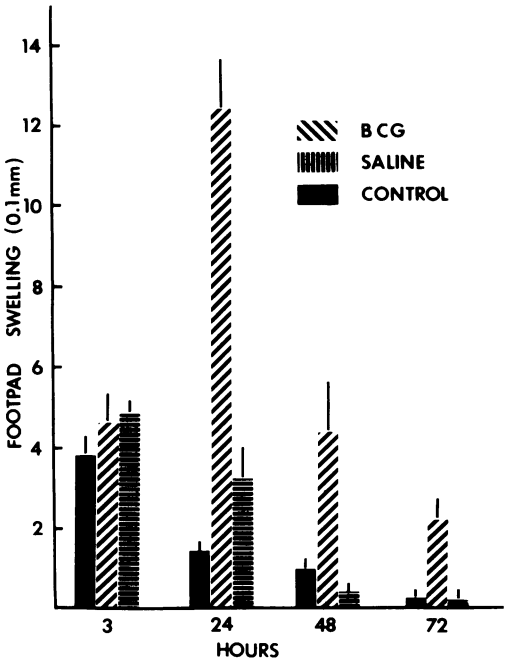

FIG. 3. *Kinetics of footpad swelling in the RHFP after a subcutaneous injection of 5 HAU of RV into mice immunized 6 days previously with 5 HAU/mouse given in the LHFP. Fourteen days before this immunization, one group of mice (BCG) received an injection of 2.6 × 10^6 living BCG in the LHFP. Another group (saline) was injected with saline in the LHFP. A third group (control) was left unimmunized and was used as a negative control for the eliciting antigen. Mean of five mice ± standard error.*

effectiveness of BCG in immunotherapy of melanoma tumors, the cross-reactivity between mycobacterial antigens and antigens from RV was tested in this system. BCG-treated mice present the same local reaction as normal mice when tested with the highest dose of RV: 2.2 ± 0.37 and 2.0 ± 0.40, respectively. When the same groups of mice were retested 6 days later with the same dose of RV, no difference in the level of DTH was observed between the two groups. This reemphasizes the necessity of injecting the BCG and the antigen into the same area drained by the same lymphoid tissue (2), and this is contrary to the theory of shared antigens being necessary for the effectiveness of BCG as an adjuvant. Although in BCG-pretreated mice the dose relationship clearly demonstrated no suppressive effect of high doses of antigen on the level of DTH, in normal mice a regulatory mechanism might operate to explain the low level of sensitivity, as described previously (17). To test this hypothesis, CY was used before immunization to prevent a possible suppressive effect on B-cell activation (12).

Immunosuppression with CY. Groups of mice were treated with CY (intravenously), and

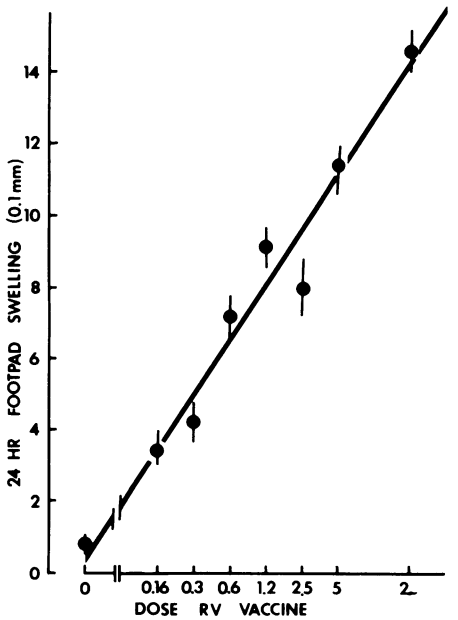

FIG. 4. *Levels of day 8 DTH developed in different groups of mice in response to varying subcutaneous eliciting doses of RV in BCG-pretreated mice immunized with 5 HAU of rabies antigen in the LHFP. Mean of five mice ± standard error.*

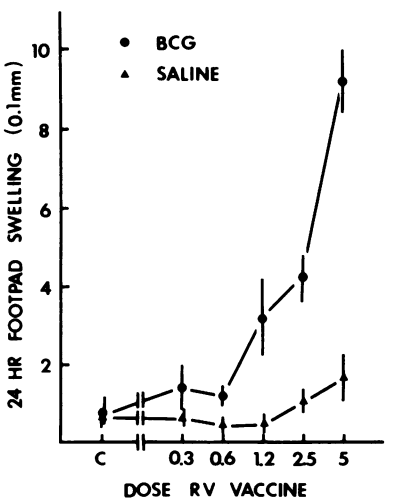

FIG. 5. *Levels of day 8 DTH developed in different groups of normal (●) or BCG-pretreated (▲) mice in response to varying doses of RV given for immunization. All mice received the same dose of eliciting antigen on day 8, and footpad swelling was measured 24 h later. Mean of five mice ± standard error.*

the same day they received varying dilutions of RV subcutaneously in the LHFP (Fig. 6); in contrast with preceding results using sheep erythrocytes (12), no such potentiation of DTH reaction occurred with RV in CY-pretreated mice immunized 8 days previously; moreover, a slight decrease was observed.

Kinetics of DTH reaction. Normal and BCG-infected mice were immunized with the highest dose of RV, as described above, and were tested with 5 HAU of RV in the RHFP every 2 days for the first week and at weekly intervals thereafter. Results of the appearance of the DTH reaction and its longevity are shown in Fig. 7. As seen in Fig. 3, BCG-preinfected mice developed a higher and more prolonged DTH reaction, and its development persisted even for 4 weeks. From the preceding experiments (Fig. 1), one can observe that RV possesses good immunogenicity, which induces a lymphoproliferative response, and effector cells can be detected with a DTH reaction at the periphery, but responses were of low magnitude. To study the capacity of this antigen to produce a cellular reaction, histology and monocyte recruitment were studied in the next experiment.

Histology and monocyte recruitment. Local reaction measured by footpad swelling is the result of specific accumulation of mononuclear cells (Fig. 8). To measure the specific recruitment of newly formed blood monocytes into the antigen-injected site, the ear test described by Miller et al. (20) was used; results were compared with footpad swelling in a concomitant experiment (Fig. 9). Comparative results were thus observed between these two methods. DTH levels observed with the footpad test in immune

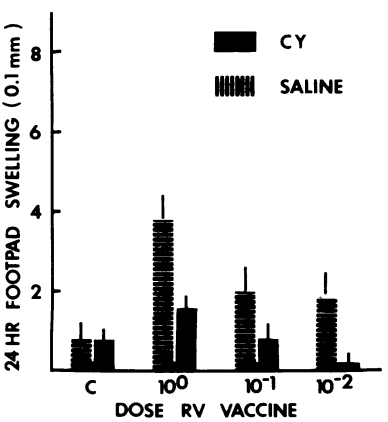

FIG. 6. *Levels of day 8 DTH developed in response to varying subcutaneously injected doses of RV in normal mice (saline) or in CY-treated mice (CY). Control mice (C) received CY and sucrose only. Mean of five mice ± standard error.*

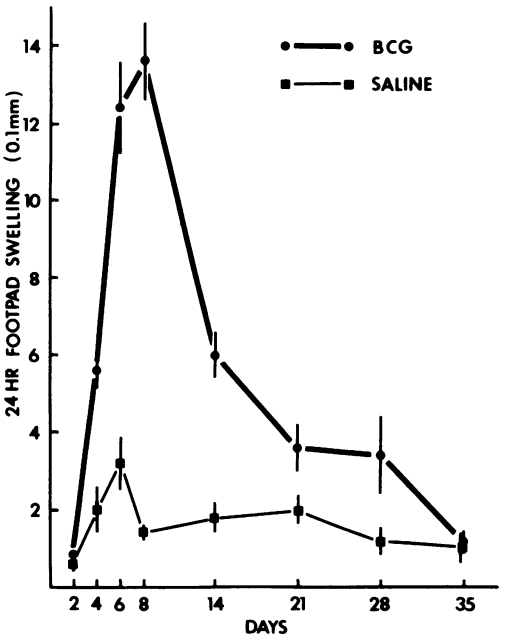

FIG. 7. *Development and decay of DTH to RV (5 HAU/mouse) in normal saline-treated mice (■) and in mice injected (subcutaneously) with 2.6 × 10^6 Pasteur strain BCG 14 days prior to immunization with RV (5 HAU/mouse) (●). Tests for DTH were performed on the day indicated; footpad swelling was read 24 h later. Mean of five mice ± standard error.*

mice related to normal mice were highly significant ($P < 0.001$), and the ear test showing preferential accumulation of newly formed labeled monocytes gave a similar significant difference between normal and immune mice ($P < 0.05$). Another argument in favor of CMI for DTH is the capacity of this reaction to be transferred into normal recipient animals with the lymphoid cells from immune donors and not with the serum of the same immune donors. The next experiments were done to examine these effector cells.

Adoptive CMI. No difference in footpad size increases were seen at 4 or 24 h (Fig. 10) between two groups of recipient mice receiving immune or control serum. Other groups of BCG-pretreated mice were injected with RV or saline in the LHFP and RHFP; they were killed 6 days later, spleen or popliteal lymph nodes were harvested, and single lymphoid cell suspensions were prepared. These cells from immune and normal mice were then tested for adoptive immunity by a systemic or local transfer in normal recipient mice. Significant 24-h footpad swelling occurred in recipient mice after both the systemic transfer and the local transfer, which gave the higher response (Fig. 11). Spleen cells from

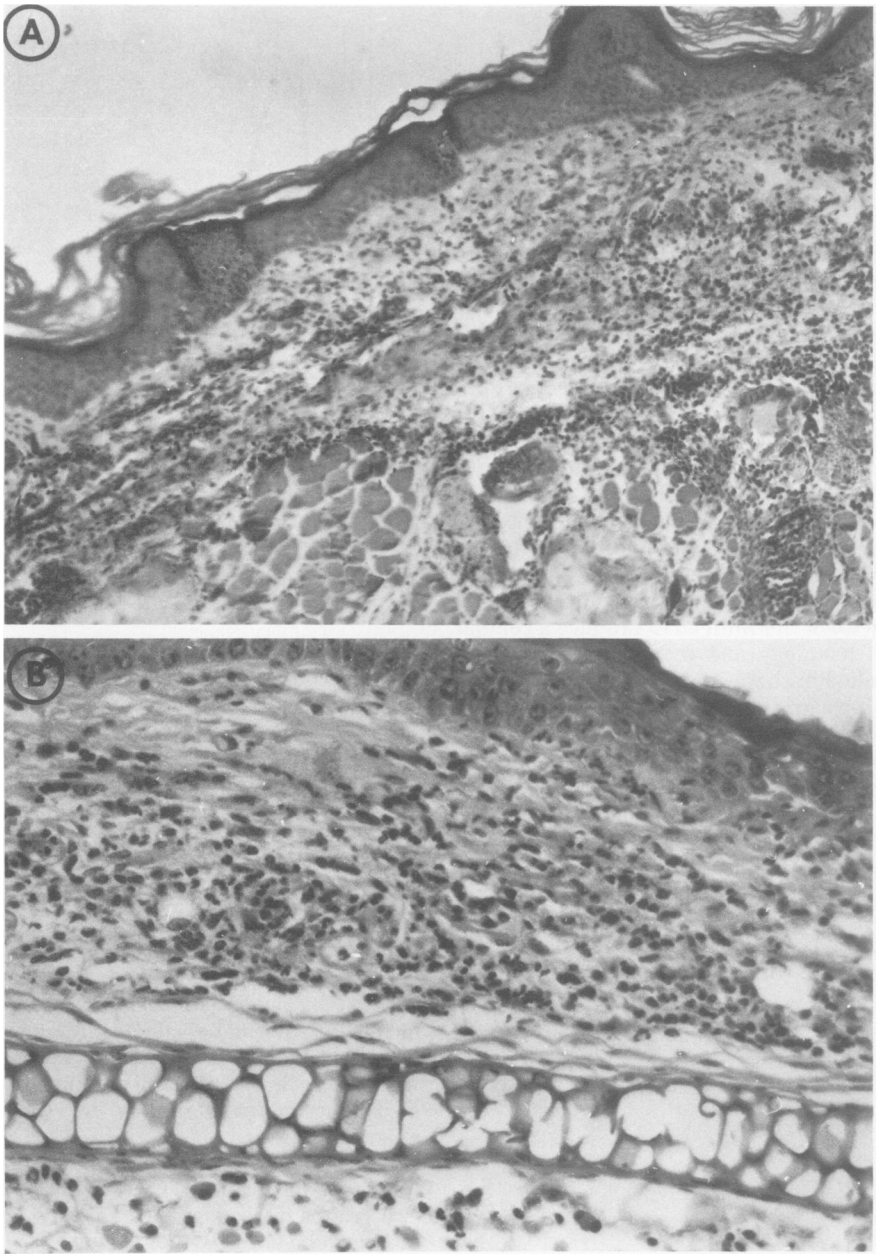

FIG. 8. *Histological appearance of the 24-h reaction elicited by 5 HAU of RV per mouse in BCG mice immunized 6 days previously with 5 HAU of RV. (A) Footpad test (×100). (B) Ear test (×200). A preponderance of mononuclear cells is seen infiltrating the cutaneous tissue (hematoxylin and eosin staining).*

immune donors did not perform as well as lymphoid cells from the same donors, but this is not statistically significant.

DISCUSSION

The footpad test has been used in mice for the assay of DTH for various replicating and non-replicating antigens. Expression of DTH did afford an in vivo demonstration of CMI in experimental infections with intracellular parasites (15) in tumor-bearing animals (24) and against histocompatibility antigens in organ transplantation (6).

The present study describes the acquired ca-

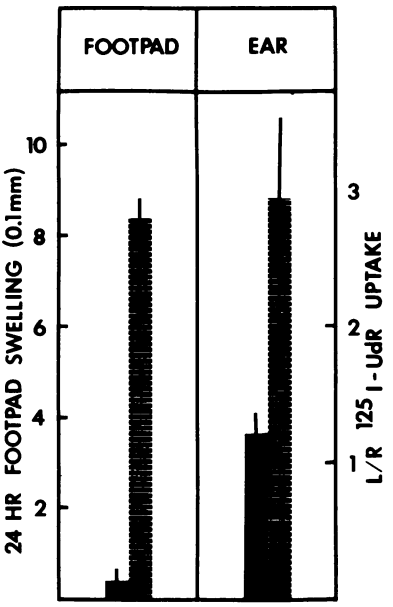

FIG. 9. *Comparison of levels of day 8 DTH in BCG-pretreated immune mice (striped bar) or in normal control mice (solid bar) elicited with 5 HAU of RV per mouse and measured at 24 h by a footpad test or an ear test, using [^{125}I]UdR-labeled monocytes. Means of six mice ± standard error.*

pacity in rabies-vaccinated mice to develop DTH after challenge with inactivated rabies virus. The consistent reproducibility of the footpad test, the kinetics of the local reaction and its association with lymphoproliferation in the draining node, and the histological appearance of the lesion and its infiltration with newly formed blood monocytes are all indicative of a DTH reaction. The ability to transfer this reactivity with cells rather than with serum also favors this hypothesis. Thus, in normal mice RV is able to induce DTH measured by the footpad test. This is in agreement with published results on evaluation of CMI by in vitro tests (23, 30, 31).

Normal mice also produced specific neutralizing antibodies after vaccination (27), and since it has been observed that antibody production can interfere with the expression of DTH mediators (17), it was of interest to use selective suppressors of B-cell response to increase the low levels of DTH observed in normal vaccinated mice. CY injected in mice before rabies vaccination did not induce DTH potentiation; on the contrary, diminished levels of DTH were observed when compared to those found in normal vaccinated mice (Fig. 6). These results are not too surprising since immunodepression has been described in tuberculin-sensitized animals (4), in methylated bovine serum albumin (7), and after inoculation of an inactivated murine mammary tumor virus in mice (unpublished data). These antigens were described as producing T-cell immunization, which is independent of concomitant B-cell response (4). This confirms the observations made by Kaplan et al. (5) and Tsiang and Atanasiu (26) in which neutralizing antibodies and protection were suppressed when mice received CY after rabies virus vaccination.

Since DTH levels were of low magnitude in normal mice after a single injection of RV, the use of a T-cell adjuvant was needed for production of higher and sustained levels of DTH, to permit easier evaluations of factors conditioning its production and expression. Because Freund complete adjuvant presents considerable side effects and its use is forbidden in humans (32), BCG vaccine, under conditions of use described elsewhere (13), was injected into mice before rabies vaccination. Under the modifying effect of BCG mice showed high and protracted levels of DTH reaction; maximum sensitization was achieved with the highest injected dose (5 HAU/mouse), and a linear dose relationship was observed between eliciting doses and levels of DTH. Potentiation of this T-cell immune response in BCG was not related to any cross-reacting antigen between BCG and RV. Current experiments indicate also that BCG potentiates the humoral immune response since an earlier and higher production of neutralizing antibodies is achieved in those BCG-pretreated mice after vaccination. This could represent an interesting modality of immunization. But instead of BCG,

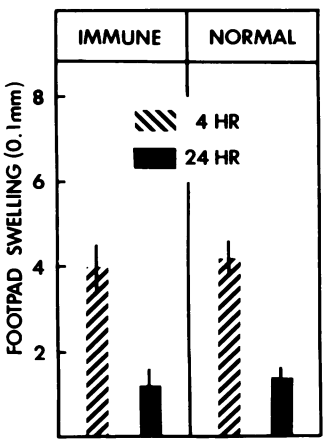

FIG. 10. *Levels of footpad swelling in normal recipient mice 4 or 24 h after intravenous injection of 0.5 ml of normal or immune mouse serum. The levels of 24-h DTH were 8.4 ± 0.24 in immune mice and 0.4 ± 0.20 in non-immunized mice. Means of five mice ± standard error.*

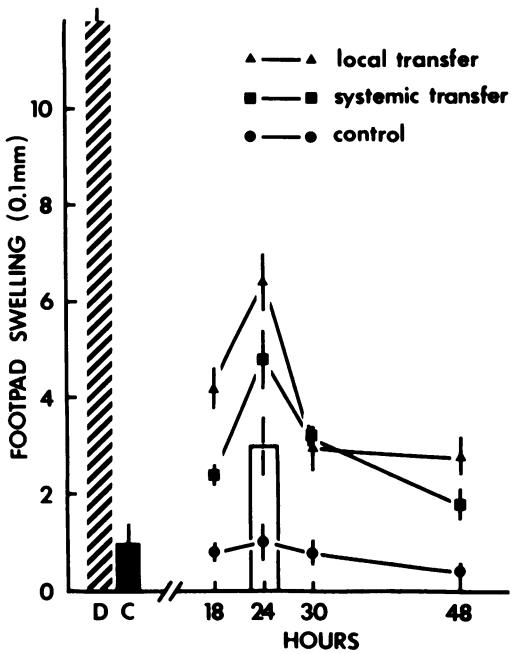

FIG. 11. *Footpad reactions in mice tested for DTH after adoptive immunization. Normal recipient mice were injected with lymph node cells from immune donors (D) either subcutaneously and mixed with the eliciting antigen (▲) or intravenously and tested simultaneously in the RHFP (■). Control recipients were normal mice receiving, subcutaneously, immune lymph node cells without the eliciting antigen (●). Normal mice injected locally with the eliciting antigen did not present a local reaction at 24 h (C). Normal recipients injected intravenously with spleen cells from immune donors and tested simultaneously with the eliciting antigen show a positive reaction (open bar). Mean of five mice ± standard error.*

other T-cell adjuvants need to be tested in order to produce high levels of specific DTH and neutralizing antibodies without any sensitization to tuberculoprotein. Induction of CMI by *N*-acetylmuramyldipeptide, for instance, associated with RV would be of great interest (32).

The present study has not determined whether or not DTH is relevant to the development of systemic protective immunity to rabies virus, although the DTH reaction has been shown to involve recruitment of newly formed blood monocytes (Fig. 9) and is described to be regularly associated with multiplication and activation of macrophages at the site of the reaction (18). Moreover, observations from Koprowski et al. (8) showed the in vitro effects of macrophages on the production of rabies virus-neutralizing antibodies, and Turner and Ballard (29) pointed out the important action of macrophages in limiting the spread of rabies infection in mice, although virus replication did not occur in peritoneal macrophages. These results could therefore offer evidence in support of an existing hypothesis (28) that CMI induced by RV is T-cell mediated through a direct action of cytotoxic T cells or by an indirect effect by macrophage stimulation. Thus, CMI against rabies virus could be detected in mice by an in vitro cytotoxicity test (30) and an in vivo DTH reaction.

Demonstration of DTH in experimental rabies infection will provide methods of determining the level of CMI developing in infected animals. Since expression of DTH can be measured easily in 24 h, the immunological status of the infected animals can be followed at precise times throughout the infection. Postchallenge repeated RV injections have been shown to produce protection in infected animals if the vaccines were able to induce interferon. It will be of great interest to evaluate also in vivo and in vitro parameters of CMI in these animals.

The ability to transfer DTH by immune cells to normal mice will be of immense value in analyzing the mechanisms of CMI when compared with the effectiveness of the neutralizing antibody transfer in rabies virus-infected mice. It must be remembered, however, that although DTH may reflect the development of CMI mechanisms, it does not necessarily reflect the development of protection against rabies virus infection. Current experiments designed to examine resistance directly are being conducted at the present time in an attempt to determine whether a correlation between DTH and protective immunity exists in vaccinated mice.

Furthermore, this may allow the development of optimal vaccination schedules. Since specific reactivity can be detected after rabies vaccination, the footpad test to RV can be used in correlation with the dosage of neutralizing antibodies and with in vitro tests, such as the cytotoxicity or lymphocyte transformation test, or lymphokine production. Adoption of such protocols will depend finally on the demonstration that DTH to rabies virus antigen is correlated with acquired protection.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Atanasiu, P., H. Tsiang, P. Perrin, S. Favre, and J. Sisman.** 1974. Extraction d'un antigène soluble (glycoprotéine) par le Triton X 100 à partir d'un vaccin antirabique de culture tissulaire de premier explant. Résultats d'immunisation et pouvoir protecteur. Ann. Microbiol. (Paris) **125B**:539-557.
2. **Delorme, E. J., J. Hodgett, J. G. Hall, and P. Alexander.** 1969. The cellular immune response to primary

sarcoma in rats: 1. The significance of large basophilic cells in thoracic duct lymph following antigenic challenge. Proc. R. Soc. London Ser. B **174**:229–236.

3. **Fleer, A., M. V. D. Hart, B. J. T. Blok-Schut, and P. T. A. Schellekens.** 1976. Correlation of PPD and BCG induced leucocyte-migration inhibition, delayed cutaneous hypersensitivity, lymphocyte transformation in vitro and humoral antibodies to PPD in man. Eur. J. Immunol. **6**:163–167.
4. **Jokipii, A. M. M., and L. Jokipii.** 1973. Suppression of cell mediated immunity by cyclophosphamide: its independence of concomitant B cell response. Cell. Immunol. **9**:477–481.
5. **Kaplan, M. M., T. J. Wiktor, and H. Koprowski.** 1975. Pathogenesis of rabies in immunodeficient mice. J. Immunol. **114**:1761–1765.
6. **Kerckhaert, J. A., G. J. V. D. Berg, and F. M. A. Hofhuis.** 1974. Influence of cyclophosphamide on the delayed hypersensitivity in the mouse after immunization with histocompatibility antigen. J. Immunol. **113**:1801–1806.
7. **Kerckhaert, J. A. M., G. J. V. D. Berg, and J. M. N. Willers.** 1974. Influence of cyclophosphamide on the delayed hypersensitivity of the mouse. Ann. Immunol. **125**:415–426.
8. **Koprowski, H., P. Mocarelli, and T. J. Wiktor.** 1972. Antibody response in vitro to an animal virus: production of rabies virus neutralizing antibodies by mouse cells in culture. Proc. Natl. Acad. Sci. U.S.A. **69**:2433–2436.
9. **Kruger, J., and R. K. Gershon.** 1972. DNA synthesis response of thymocytes to a variety of antigens. J. Immunol. **108**:581–585.
10. **Lagrange, P. H., and G. B. Mackaness.** 1975. A stable form of delayed type hypersensitivity. J. Exp. Med. **141**:82–96.
11. **Lagrange, P. H., G. B. Mackaness, and T. E. Miller.** 1974. Influence of dose and route of antigen injection on the immunological induction of T cells. J. Exp. Med. **139**:528–542.
12. **Lagrange, P. H., G. B. Mackaness, and T. E. Miller.** 1974. Potentiation of T-cell mediated immunity by selective suppression of antibody formation with cyclophosphamide. J. Exp. Med. **139**:1529–1539.
13. **Lagrange, P. H., G. B. Mackaness, and T. E. Miller.** 1976. Parameter conditioning the potentiating effect of BCG on the immune response, p. 23–36. *In* G. Lamoureux, R. Turcotte, and V. Portelance (ed.), BCG in cancer immunotherapy. Grune and Stratton, New York.
14. **Mackaness, G. B.** 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med. **129**:973–992.
15. **Mackaness, G. B.** 1971. Delayed type hypersensitivity and the mechanism of cellular resistance to infection, p. 413–424. *In* B. Amos (ed.), Progress in immunology. Academic Press Inc., New York.
16. **Mackaness, G. B., D. J. Auclair, and P. H. Lagrange.** 1973. Immunopotentiation with BCG. I. Immune response to different strains and preparations. J. Natl. Cancer Inst. **51**:1655–1667.
17. **Mackaness, G. B., P. H. Lagrange, T. E. Miller, and T. Ishibashi.** 1974. Feedback inhibition of specifically sensitized lymphocytes. J. Exp. Med. **139**:543–559.
18. **Mackaness, G. B., P. H. Lagrange, T. E. Miller, and T. Ishibashi.** 1974. The formation of activated T cells, p. 193–208. *In* W. H. Wagner and H. Hahn (ed.), Activation of macrophages, vol. 2. Excerpta Medica, Amsterdam.
19. **Metaxas, M. N., and M. Metaxas-Buehler.** 1955. Studies on the cellular transfer of tuberculin sensitivity in the guinea pig. J. Immunol. **75**:333–347.
20. **Miller, J. F. A. P., M. A. Vadas, A. Whitelaw, and J. Gamble.** 1975. A radioisotopic method to measure delayed type hypersensitivity in the mouse. II. Cell transfer studies. Int. Arch. Allergy **49**:639–708.
21. **Miller, T. E., G. B. Mackaness, and P. H. Lagrange.** 1973. Immunopotentiation with BCG. II. Modulation of the response to sheep red blood cells. J. Natl. Cancer Inst. **51**:1669–1675.
22. **Minden, P., T. R. Sharpton, and J. K. McClatchy.** 1976. Shared antigens between human malignant melanoma cells and *Mycobacterium bovis* (BCG). J. Immunol. **116**:1407–1414.
23. **Nozaki, J., and P. Atanasiu.** 1976. Etude comparative in vitro des antigènes rabiques et des virus apparentés à la rage par la technique des lymphocytes sensibilisés. Ann. Microbiol. (Paris) **127A**:429–438.
24. **Peters, R. L., R. M. Donahoe, and G. I. Kelloff.** 1975. Assay in the mouse for delayed type hypersensitivity to murine leukemia virus. J. Natl. Cancer Inst. **55**:1089–1095.
25. **Thursh, D. R., and E. E. Emerson.** 1971. Selective DNA synthesis by cells specifically localizing in response to xenogeneic erythrocytes. J. Exp. Med. **138**:659–671.
26. **Tsiang, H., and P. Atanasiu.** 1975. Effets de la cyclophosphamide sur l'infection rabique chez la souris. Protection conférée par la sérothérapie. C. R. Acad. Sci. **281**:957–960.
27. **Turner, G. S.** 1973. Humoral and cellular immune response of mice to rabies and smallpox vaccines. Nature (London) New Biol. **241**:90–92.
28. **Turner, G. S.** 1976. Thymus dependence of rabies vaccine. J. Gen. Virol. **33**:535–538.
29. **Turner, G. S., and R. Ballard.** 1976. Interaction of mouse peritoneal macrophages with fixed rabies virus in vivo and in vitro. J. Gen. Virol. **30**:223–231.
30. **Wiktor, T. J., P. C. Doherty, and H. Koprowski.** 1977. In vitro evidence of cell-mediated immunity after exposure of mice to both live and inactivated rabies virus. Proc. Natl. Acad. Sci. U.S.A. **74**:334–338.
31. **Wiktor, T. J., I. Kamo, and H. Koprowski.** 1974. In vitro stimulation of rabbit lymphocytes after immunization with live or inactivated vaccines. J. Immunol. **112**:2013–2019.
32. **World Health Organization.** 1976. Immunological adjuvant. Tech. Rep. Ser. 595. World Health Organization, Geneva.