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Kinetics of Different Specific Immunological Parameters After Rabies Vaccination in Mice

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A significant protection to an intracerebral challenge of 70 mean lethal doses of a standard live rabies virus strain was obtained in BCG-pretreated mice or in normal mice which had been immunized with a single subcutaneous injection of a beta-propiolactone-inactivated rabies vaccine. Concomitantly, levels of delayed-type hypersensitivity (measured in vivo by the footpad test) and serum-neutralizing activity were evaluated at various times after immunization. All immune criteria were significantly augmented in the BCG-pretreated, rabies-immune mice as compared to normal, rabies-immune mice. However, peak levels of protection, delayed-type hypersensitivity, and serum-neutralizing activity did not occur at the same times. For instance, in the BCG-pretreated, rabies-immune mice, delayed-type hypersensitivity peaked on day 7, protection peaked on day 21, and serum-neutralizing activity peaked on day 60. In BCG-pretreated mice, which did not receive the rabies vaccine, positive delayed-type hypersensitivity, some protection, and serum neutralizing activity were observed 4 to 5 weeks after BCG pretreatment. The possible relationships between specific and nonspecific immunity provoked by rabies virus antigens, tissue culture cell-associated antigens (derived from the bovine fetal kidney cells in which the rabies virus was grown), and BCG are discussed.

The roles of humoral and cell-mediated immunity (CMI) have been well-defined in infections of experimental animals (2, 6) and in humans (1). The mechanisms involved in acquired resistance after natural or experimental infection with rabies virus are still a matter of controversy even if previous reports have made clear the distinction between the two mechanisms. Since antibody-neutralizing activity can be tested easily, great emphasis has been placed on the role of humoral immunity after rabies vaccination. There is indirect proof that CMI exists in rabies-vaccinated mice: vaccine had no protective effect on thymus-less mice (24). In recent years, direct proofs for the existence of CMI has been assayed in vitro and in vivo. CMI was evaluated in vitro by using cytotoxicity tests (26) or blast transformation (3, 27; H. T. Hill, Diss. Abst. Int. B **35:** 571, 1975). In vivo, CMI was evaluated by using the footpad test assay after vaccination with inactivated rabies virus vaccine (15). This local reaction has all the characteristics of a delayed-type hypersensitivity (DTH) reaction: kinetics, histology, and specific transfer of sensitivity by lymphocytes, but not by serum, of immune mice.

The present report deals with a comparative study between the kinetics of DTH, neutralizing-antibody activity, and acquired resistance induced after rabies vaccination in normal or in BCG-pretreated mice as previously described (15).

MATERIALS AND METHODS

Animals. A total of 1,800 specific-pathogen-free Swiss, outbred, male OF1 mice were purchased from Iffa Credo (Domaine des Oncins, Saint Germain sur l'Arbresle, France). They were 4 weeks of age at the start of each experiment.

Rabies vaccine (RV). β-Propiolactone-inactivated fixed rabies virus was prepared in bovine kidney cells and purified as previously described (5). It contained 2,560 hemagglutin units (HAU) per ml and was kept deep frozen at −70°C in small vials.

BCG vaccine. The Pasteur strain of BCG vaccine was obtained as described previously (11). In brief, commercial vaccine, obtained from the Pasteur Institute, was cultivated in Proskauer and Beck (PB) medium with 1% glucose and 0.05% Tween 80 for 7 days at 37°C in roller bottles. The mycobacteria were washed twice in fresh PB medium and homogenized by a short pulse of ultrasonication. BCG vaccine contained 4×10^8 viable units per ml and was stored deep frozen at −70°C in small vials containing 1 ml of BCG vaccine.

Immunization. Four hundred mice were divided into four equal groups, called A, B, C, and D, in 10 cages of 10 mice each.

The mice of groups A and B received one injection

of BCG vaccine (2×10^6 viable units) in the left hind footpad (LHFP) in a volume of 0.04 ml. Two weeks later, the mice of groups A and C received one injection of 5 HAU of RV in the LHFP in a volume of 0.04 ml. The control group, the mice of group D, received the same volume of saline in the LHFP. In 7, 14, 21, and 60 days after the injections, 25 mice of each group were tested for DTH, serum-neutralizing activity, and resistance to living rabies virus challenge.

Challenge with rabies virus. At each time point, 10 mice were challenged by an intracerebral injection of 0.03 ml containing 70 mean lethal doses (LD_{50}) of the standard strain of rabies virus (CVS). On day 60 after immunization, the challenge dose was readjusted to compensate for the natural increase in resistance of older mice. This was done by reevaluation of the LD_{50} challenge dose in age-matched nonimmune mice. Neurological symptoms, such as hyperexcitability or paralysis, and death were recorded at daily intervals for individual mice. Mice living more than 30 days after the 70 LD_{50} challenge were recorded as resistant.

The mean survival time after challenge was evaluated graphically for the time at which 50% of the nonresistant mice were dead.

DTH. At each time point after immunization, five mice per group were tested by using the footpad test as previously reported (15). In brief, 0.04 ml of RV containing 5 HAU was injected into the right hind footpad (RHFP), and increased footpad thickness was measured, with a gauge caliper (Schnelltaster, System Kröplin, 0.05 mm) at various times thereafter.

Serum antibodies. At each time point, 10 mice per group were bled and the sera were pooled into two groups of sera from 5 mice. The levels of specific antibody were evaluated by using two methods. The first one was the sero-neutralization test as described in the World Health Organization monograph on rabies (28). The second one was an assay of the protective effect of different dilutions of immune serum ($10^{-0.7}$, $10^{-1.4}$, $10^{-2.1}$, and $10^{-2.8}$). Various dilutions of serum were injected intravenously 18 h before 100 LD_{50} challenge was given intramuscularly in a volume of 0.2 ml. A positive control serum was also used concomitantly to allow the protective index to be expressed in international units.

RESULTS

Kinetics of acquired resistance after rabies vaccination. When normal mice were challenged with an intracerebral injection of 70 LD_{50}, early neurological symptoms occurred within a few days, and mice died 1 to 3 days later. When mice pretreated with RV, BCG vaccine, or both, were challenged at various times thereafter, the occurrence of clinical neurological symptoms in nonresistant mice did not differ from the controls. But when the mean survival time was evaluated in nonresistant mice, the survival time was always significantly prolonged in specifically preimmunized mice whether or not mice were pretreated with BCG (Table 1). When the definitive resistance was evaluated, a striking difference was seen between these two groups, as shown in Fig. 1, which reports the percentage of resistance 30 days after challenge. Maximum protection was achieved 3 weeks after rabies vaccination in BCG-pretreated mice and persisted to some extent even after 60 days. It is noteworthy that BCG-pretreated, nonimmunized mice also showed some acquired resistance. The best nonspecific activity occurred 4 to 5 weeks after subcutaneous injection of BCG.

TABLE 1. *Mean of mortality times after intracerebral challenge with 70 LD_{50} of CVS in different groups of susceptible mice at various times after immunization*

Group	Time of death (h)[a] after immunization at day:				
	7	14	21	60	Mean
A	204	271	245	199	229.7
B	185	180	206	180	187.7
C	194	250	264	180	222
D	185	180	180	180	181.2

[a] This mean mortality time after challenge was evaluated graphically when 50% of nonresistant mice were dead.

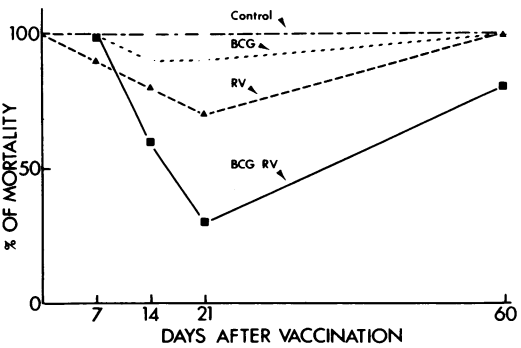

FIG. 1. *Percentage of mortality recorded 30 days after intracerebral challenge with 70 LD_{50} of CVS in different groups of 10 mice at various times after immunization.*

Kinetics of the specific CMI response after vaccination. The time course of the DTH reaction was recorded at various times after vaccination (Fig. 2). As reported previously (15), DTH elicited with 5 HAU of the RV was at its maximum 7 days after immunization and peaked 24 h after elicitation. The levels of DTH were higher in BCG-pretreated RV-immunized mice than in normal RV-immunized mice, this difference being statistically highly significant ($P < 0.01$). But unexpected results were observed when BCG-pretreated non-RV-immunized mice were tested with RV vaccine. The 24-h reactions to RV antigen were observed to be higher and statistically significant in BCG-pretreated mice as compared to control mice ($P < 0.05$).

Kinetics of the specific humoral response after vaccination. The production of rabies virus neutralizing antibodies in pooled sera from

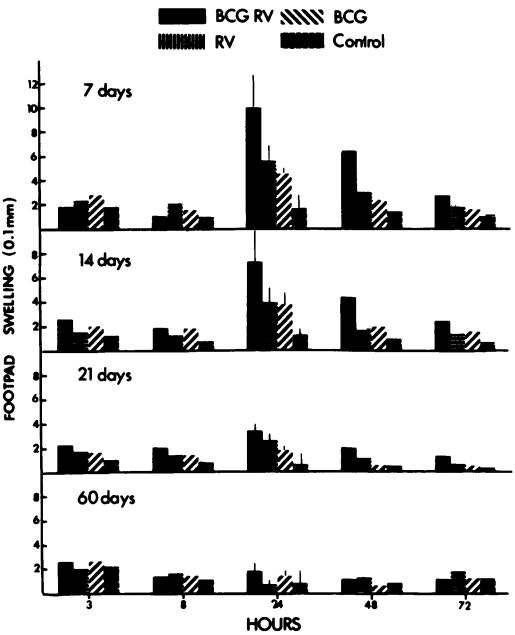

FIG. 2. *Time course of DTH reactions measured at varying times after immunization in different groups of mice. Mean of 5 mice ± standard error of the mean.*

each group was evaluated at various time intervals after vaccination, and the protective index was expressed in international units by using a positive control serum.

The neutralizing antibody production increased during the entire observation period in BCG-RV-immunized mice, raising a very high titer, if we consider the single immunizing dose of 5 HAU (Table 2). In the RV-immunized mice, the peak level was reached on day 21 and was at a very low level on day 60. And when the comparison between BCG-RV- and normal RV-immunized mice was made on day 21 postvaccination, a higher titer was noticed in the normal RV-immunized mice than in BCG-RV-immunized mice (one must also consider that some neutralizing activity was produced in the serum of BCG-pretreated non-RV-vaccinated mice). This neutralizing activity was observed from day 7 to day 60 of the observation period which began 2 weeks after BCG pretreatment.

When pooled sera were used to evaluate the in vivo protective activity of the serum from various groups of mice, the results were not clear cut because of the very low titers of neutralizing antibody in the serum. In fact, the serum protective effect follows a direct linear relationship between dose and effect only when high titers of antibody (more than 10 IU/ml) are present in the serum. This was never seen in our experiments.

Comparison of the kinetics of the different immune parameters. The time courses of immune parameters after viral or bacterial infection have been used by different researchers (6, 21) to evaluate the relationships between the different immune parameters. Figure 3 represents a composite picture of the acquired resistance to 70 LD_{50} intracerebral challenge, the DTH levels and the neutralizing activity of the serum measured at various time intervals after immunization in different groups of mice.

The DTH reactions always appeared during the early stage after immunization, peaking on day 7 and decreasing thereafter (Fig. 3). Thus, an inverse temporal relationship seems to exist between DTH and acquired resistance because acquired resistance increases gradually from the day after immunization until day 21. There appears to be a direct relationship between levels of neutralizing antibody and acquired resistance, since increased resistance is temporally associated with increased neutralizing antibody levels. This was the case in group C of normal RV-immunized mice. But when the level of acquired resistance was compared to the level of neutralizing antibodies in the different groups of mice, some differences became apparent between RV-vaccinated normal and BCG-pretreated mice. Higher protection (70%) was seen in BCG-pretreated RV-immunized mice, in which the level of neutralizing antibody was much lower than in

TABLE 2. *Serum[a] neutralizing titer in different groups of mice at various days after immunization*

Group[b]	Titer at day after immunization:			
	7	14	21	60
A				
1	0.11	0.82	1.16	5.90
2	0.10	1.30	1.83	4.64
Mean	0.105	1.06	1.49	5.27
B				
1	0.023	0.03	0.00	0.03
2	0.053	0.00	0.02	0.019
Mean	0.038	0.015	0.010	0.024
C				
1	0.10	1.32	2.90	0.29
2	0.14	0.37	2.32	0.43
Mean	0.12	0.84	2.61	0.36
D				
1	0	0	0	0
2	0	0	0	0
Mean	0	0	0	0

[a] Neutralization titer was expressed in international units by comparing efficacy of protection using an international neutralizing serum.

[b] Pools of serum of five mice were used, and serum from 10 mice per group were obtained.

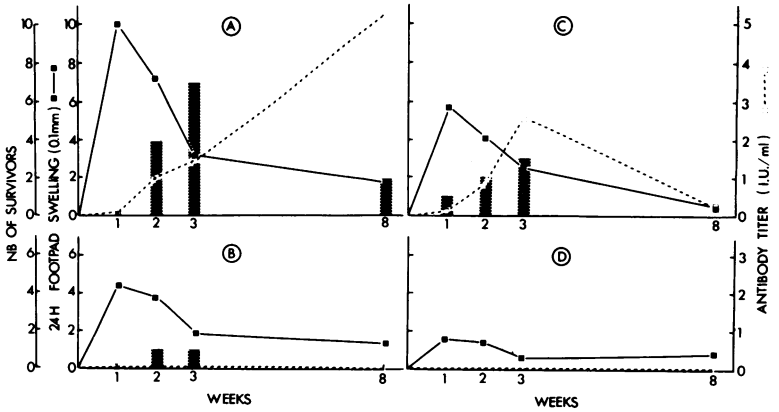

FIG. 3. *Kinetics of 24-h DTH reaction (■), neutralizing-antibody titers (in international units per ml) (□), and number of survivors (hatched columns) in BCG-pretreated and RV-immunized mice (A), in RV-immunized mice (C), in BCG-pretreated mice (B), or in control mice (D).*

normal RV-immunized mice in which only 30% of protection was observed.

DISCUSSION

The present report deals with the comparative studies of different parameters of the immune response after immunization with a single injection of 5 HAU of an inactivated RV. The effect of pretreatment with BCG vaccine was also looked at because of its effectiveness in promoting a stable form of CMI (12). The end purpose of these studies was to compare the kinetics of acquired resistance after vaccination and the time course of cell-mediated hypersensitivity measured in vivo by the proposed test, and the humoral levels of neutralizing activity of the serum. The latter are, at the present time, the regular tests used to evaluate effectiveness of the RV. From the reported results, some conclusions can be made.

First, the protective effect of the BCG pretreatment alone was unexpected. BCG vaccination produced DTH to rabies vaccine and also some neutralizing activity in the serum (Table 2). Its ability to produce or to amplify the levels of DTH and humoral antibodies to foreign, noncross-reacting antigen is well-established (10, 11, 14–19). Its action on the natural resistance to rabies infection has not been described previously. The relation of this with the nonspecific increase in resistance to virus infections observed after BCG vaccination (14) should be investigated. Since some protection was observed after intracerebral challenge of 70 LD_{50}, which represents a very drastic inoculum, the protective effect of BCG vaccination should also be evaluated after subcutaneous and intramuscular challenge. More work needs to be done in order to have a more precise view on the early events in rabies infection in normal or in reticuloendothelial system-stimulated mice (25).

BCG pretreatment was also able to produce neutralizing activity in the serum (Table 2) and DTH to RV (Fig. 1). These levels were statistically significant when compared to the levels found in control mice. These facts, which could render unclear the specificity of the DTH reaction and the sero-neutralization test, point to the existence of some neutralizing activities of serum after mycobacterial infections in animals. Thus, our results are comparable with those of Srinivasan (22) in which acquired resistance to rabies infection was observed after *Mycobacterium phlei* infection. Haldar and co-workers (7) show also acquired resistance to rabies infection after injections of trypsin-treated extracts from these mycobacteria in different experimental animals. Neutralizing activities to rabies virus were also observed in sera of these pretreated animals. Explanations of these phenomena are not conclusive at the present time. Some hypotheses might be made concerning the possible role of cross-reacting antigens between the BCG vaccine and the RV vaccine. In fact, as far as DTH reactions are concerned, when a more highly purified RV (4) was used, no such cross-reactions were observed (15). Since, in the present report, RV was produced in bovine fetal kidney cell culture, one can not exclude the possibility that there are cross-reacting antigens in the BCG vaccine and the tissue culture cells, as in the case with BCG and human malignant melanoma cells (20). Since in the present case, BCG was grown in PB medium, without BSA, this antigen did not seem to be involved for the cross-reactivity. It would be worthwhile to find out the exact cross-reactivity antigen in order to avoid (or maybe to promote) any cross-reactivity be-

tween BCG and RV for medical use in patients who have been previously sensitized with mycobacterial antigens.

Because of these putative cross-reactions and its adjuvant activity, BCG pretreatment was able to promote higher levels of all specific immune parameters after vaccination with inactivated rabies virus. But the peak levels of each parameter are not matched (Fig. 3): DTH being at its maximum on day 7, acquired resistance on day 21 and serum-neutralizing activity on day 60. Thus, it seems clear that neither all specific T cells nor all in vitro specific neutralizing antibodies are involved in acquired resistance.

Since after immunization different subsets of specific T cells appeared and the DTH reactions are only able to measure the final effect, it would be interesting to test separately the different subsets of cells involved in the DTH reactions, i.e., blast cells or memory cells (12).

Moreover, other functions of T cells are known, for example cell cytotoxicity which is produced after rabies vaccination (26). The next approach will be devoted to evaluating the kinetics of such functions after RV vaccination in normal or BCG-pretreated mice. With the same reasoning an increase of antibody-dependent cell cytotoxicity should also be evaluated, which may account for the augmented acquired resistance. These different methods are currently being investigated in this laboratory.

When the different levels of the immune parameters were observed, the degree of each particular response (protection, DTH, and neutralizing antibodies) was always coordinated and varied in the same way in the different group of experimental animals. No response in control mice, some in BCG-pretreated mice, high responses in RV-immunized normal mice, with a coincident peak level of resistance and serum neutralizing activity on day 21 after vaccination; the highest responses were observed in BCG-pretreated, RV-immunized mice. It would also be interesting to compare the effectiveness of vaccination with more than one injection of RV and the adjuvant effect of BCG with only a single injection of RV. Increasing the quantity of purified RV could give a very potent, long-lasting immunity when used after BCG pretreatment (15).

There is a correlation between the protective effect of vaccine and DTH levels (as measured on day 7) and acquired resistance, although the actual mechanism of specific protection is unknown. Since the skin test is a very simple, inexpensive technique, it would be useful for people working with rabies vaccine control to be aware that DTH reaction to rabies antigens is another good, though indirect, parameter of CMI, which can measure the protective efficacy of a given commercial vaccine for prophylaxis against rabies infection.

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LITERATURE CITED

1. **Allen, J. C.** 1976. Infection complicating neoplastic disease and cytotoxic therapy, p. 151-171. *In* J. C. Allen (ed.), Infection and the compromised host. The Williams & Wilkins Co., Baltimore.
2. **Allison, A. C.** 1974. Interactions of antibodies, complement components and various cell types in immunity against viruses and pyogenic bacteria. Transplant. Rev. **19**:3-55.
3. **Atanasiu, P., J. Nozaki-Renard, V. Savy, and A. Eyquem.** 1977. Evaluation de l'immunité cellulaire après vaccination antirabique chez l'homme. C.R. Acad. Sci. Paris **285(D)**:1187-1190.
4. **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.
5. **Atanasiu, P., H. Tsiang, P. Reculard, F. Aguilon, M. Lavergne, and P. Abamovicz.** 1977. Zonal centrifuge preparation of human rabies vaccine obtained on bovine fetal kidney cells. Biological results. Dev. Biol. Stand. **40**:35-44.
6. **Blanden, R. V.** 1974. T cell response to viral and bacterial infection. Transplant. Rev. **19**:56-88.
7. **Haldar, S., S. Singh, B. P. Mallick, and B. K. Kathuria.** 1977. Nonspecific resistance against rabies virus in mice, rats and guinea-pigs. Ind. J. Exp. Biol. **15**:370-372.
8. **Ionescu, D.** 1944. Untersuchungen uber die naturliche Immunitat bie der Tollwut. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **151**:254-260.
9. **Koprowski, H., P. Nocarelli, and T. J. Wiktror.** 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.
10. **Lagrange, P. H.** 1977. Nonspecific resistance to virus infection induced by immunostimulation. Bull. Inst. Pasteur **75**:291-307.
11. **Lagrange, P. H., B. Hurtrel, and P. Ravisse.** 1978. La réaction locale granulomateuse après injection sous-cutanée de BCG chez la souris. I. Description. Ann. Immunol. (Paris) **129C**:529-546.
12. **Lagrange, P. H., and G. B. Mackaness.** 1975. A stable form of delayed-type hypersensitivity. J. Exp. Med. **141**:82-96.
13. **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.
14. **Lagrange, P. H., G. B. Mackaness, and T. E. Miller.** 1976. Parameter conditioning the potentiating effect of BCG on the immune response, p. 23-26. *In* G. Lamoureux, R. Turcotte and V. Portelance (ed.), BCG in cancer immunotherapy. Grune and Stratton, New York.
15. **Lagrange, P. H., H. Tsiang, and B. Hurtrel.** 1978. Delayed-type hypersensitivity to rabies virus in mice:

assay of active or passive sensitization by the footpad test. Infect. Immun. **21**:931-939.

16. **Loffler, E., and F. Schweinburg.** 1934. Zur Theorie der Immunitat bei Tollwut. Virchows Arch. Pathol. Anat. Physiol. **283**:540-549.
17. **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.
18. **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.
19. **Mackaness, G. B., P. H. Lagrange, and T. Ishibashi.** 1974. The modifying effect of BCG on the immunological induction of T cells. J. Exp. Med. **139**:1540-1552.
20. **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.
21. **North, R. J., G. B. Mackaness, and R. W. Elliot.** 1972. The histogenesis of Immunologically committed lymphocytes. Cell. Immunol. **3**:680-694.
22. **Srinivasan, V. A., B. B. Mallick, and B. K. Kathuria.** 1973. Studies on the nonspecific resistance against rabies virus as induced by single and multiple inoculations. Aspects Allergy Appl. Immunol. **6**:231-234.
23. **Turner, G. S.** 1973. Humoral and cellular immune response of mice to rabies and smallpox vaccines. Nature (London) New Biol. **241**:90-92.
24. **Turner, G. S.** 1976. Thymus dependence of rabies vaccine. J. Gen. Virol. **33**:535-538.
25. **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.
26. **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.
27. **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.
28. **World Health Organization.** 1973. Laboratory techniques in rabies. W.H.O. Monogr. Ser. 23.