Proc. Natl. Acad. Sci. USA
Vol. 74, No. 1, pp. 334–338, January 1977
Immunology

In vitro evidence of cell-mediated immunity after exposure of mice to both live and inactivated rabies virus

(rabies vaccine/thymus-derived cells/immunosuppression/*H-2* genes/β-propiolactone)

T. J. WIKTOR, P. C. DOHERTY, AND H. KOPROWSKI

The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, Pennsylvania 19104

Contributed by Hilary Koprowski, October 12, 1976

ABSTRACT **Mice exposed to live or β-propiolactone-inactivated rabies virus generated a strong, specific cell-mediated cytotoxic response which was generally maximal 6 days after inoculation. Release of ^{51}Cr was apparently a function of immune thymus-derived lymphocytes (T cells) because it was abrogated by prior incubation of spleen cells with anti-thymus antiserum and complement but was undiminished by passage of spleen cells through nylon-wool columns. Cytotoxicity was always maximal for interactions in which thymus-derived cells and targets shared *H-2* genes but, unlike the situation found in other assays of this type, considerable lysis of allogeneic, virus-infected target cells may also occur. Perhaps the most significant finding from these experiments is that an inactivated virus has been shown to stimulate a potent cytotoxic thymus-derived cell response. Manipulation of this experimental model may allow analysis of the antigens required for stimulation of cell-mediated immunity. A more practical consequence may be the development of more rational protocols for postexposure vaccination against rabies. Prior treatment of mice with antirabies antibody severely depressed the generation of cell-mediated immunity.**

The possible role of cell-mediated immunity in rabies infection is poorly understood (1–3) but is worth investigating. Rabies, because of its prolonged incubation period, is the only virus disease in which postexposure vaccination is routinely practiced. However, despite the fact that such postexposure treatment has been applied since 1885 (4), the mechanisms underlying its mode of action have remained obscure. In addition, treatment failures are not uncommon (5, 6) and there is no experimental basis for determining optimal immunization schedules for man. Analysis of the cellular immune response in rabies may elucidate some of the critical events occurring in rabies virus-host cell interactions and help in establishing the most effective protocol for postexposure treatment.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), A/J, BALB/c, CBA/J, C57BL/10 (B10), B10.A, and B10.A (5R) inbred mice were purchased from the Jackson Laboratories, Bar Harbor, Maine.

Virus Preparations. The ERA strain of rabies virus (7) was plaque-purified and grown in BHK21 cells (8); the infectivity titer of the virus stocks was $10^{8.0}$ plaque-forming units (PFU)/ml. The same ERA virus was used as an inactivated vaccine after 20-fold concentration by ultrafiltration and inactivation by β-propiolactone (8). The antigenicity of this vaccine preparation, as evaluated by the standard potency test in mice (9), was 25 times that of reference National Institutes of Health vaccine lot 180.

Adult (8- to 10-week-old) mice were generally inoculated intraperitoneally with $10^{7.0}$ PFU of live ERA virus or with 0.1 ml of undiluted β-propiolactone-inactivated vaccine, and immune spleen cells were assayed 6 or 7 days later.

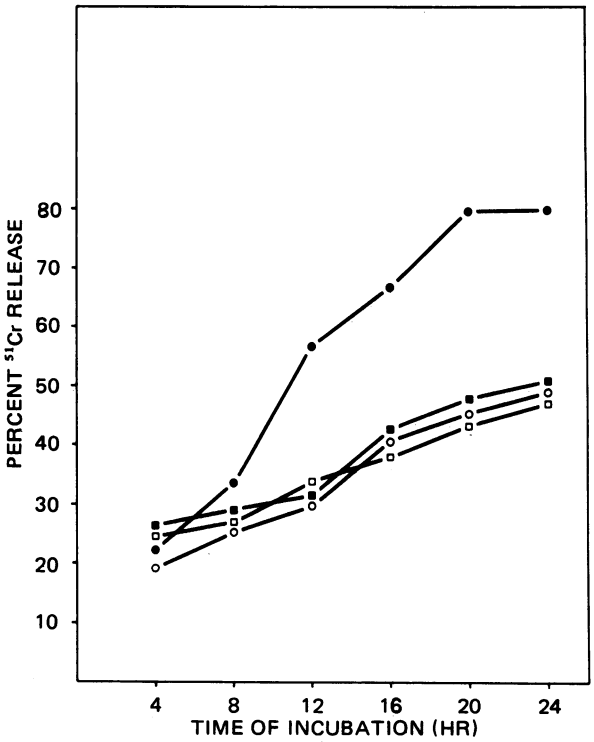

FIG. 1. Time course of ^{51}Cr release from rabies virus-infected (○ ●) and control (□ ■) MNB target cells caused by immune (● ■) or normal (○ □) spleen cells, overlaid at a ratio of 100:1. Syngeneic A/J mice were immunized intraperitoneally with $10^{7.0}$ PFU of live ERA rabies virus 7 days prior to assay. Results are presented as total % ^{51}Cr release (17), to show background levels of lysis.

Specificity experiments were done with the Westminster (WE3) strain of lymphocytic choriomeningitis virus (LCMV), using techniques described previously (10).

Processing of Lymphocytes. Single cell suspensions of spleen were prepared by standard methods and erythrocytes were removed by treatment with NH_4Cl (11). Depletion of thymus-derived cells (T cells) was achieved by treatment with antithymus antiserum (anti-Thy-1 serum; AKR/J anti-C3H/HeJ, Bionetics, Kensington, Md.) and guinea pig complement (12); B cells were removed by passage through nylon-wool columns (13).

Target Cells. A neuroblastoma cell line of A/J (*H-2*^{a}) origin (MNB) (14) (kindly supplied by E. M. Levine, Wistar Institute) and a fibrosarcoma cell line derived from B6 (*H-2*^{b}) mice (MC57G) (15) (generously supplied by B. B. Knowles, Wistar Institute) were found suitable as target cells—i.e., they were

Abbreviations: PFU, plaque-forming units; LCMV, lymphocytic choriomeningitis virus; T cells, thymus-derived cells; EMEM10, Eagle's minimal essential medium with 10% fetal bovine serum.

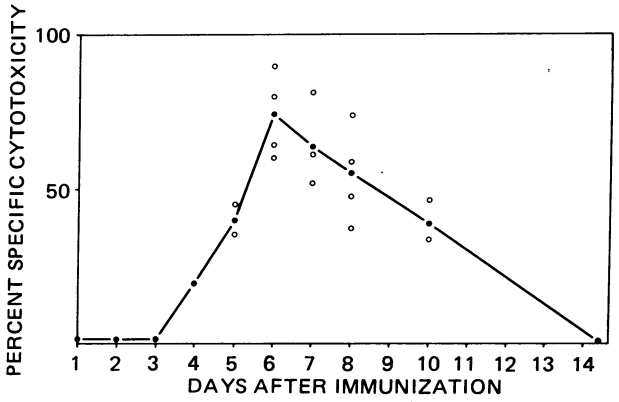

FIG. 2. Development of cell-mediated cytotoxicity after intraperitoneal immunization with $10^{7.0}$ PFU of live ERA virus. (○), results of separate experiments; ●, mean value of indicated day's experiments. These results are for the syngeneic A/J, MNB lymphocyte-target system (*H-2*^{a}). The data for the B6-MC57G interaction (unpublished) were identical.

readily infected with rabies virus and showed immunofluorescent evidence (16) of surface viral antigen expression but did not develop cytopathic effects during the course of the assay as a result of virus infection.

Trypsinized target cells (5×10^6) were incubated at 37° for 10 min with ERA rabies virus, at a concentration of 5 PFU per cell. They were then diluted in 6 ml of Eagle's minimal essential medium supplemented with 10% fetal bovine serum (EMEM10) and seeded in plastic bottles (T25 Falcon 3013). After incubation for 20 hr at 37° they were trypsinized again, taken up in 10 ml of medium, and sedimented at $150 \times g$. Cells were then resuspended in 1 ml of EMEM10 containing 200 μCi of ^{51}Cr ($Na_2{}^{51}CrO_4$, New England Nuclear, Boston, Mass.) and incubated for 60 min at 37°. They were then washed twice in 50 ml of EMEM10 and dispensed into flat-bottomed, 96-hole tissue-culture trays (Microtest II Tissue Culture Plates, Falcon 3040) at a concentration of 2×10^4 cells per well in 50 μl of medium. Uninfected target cells were labeled and dispensed in the same way for use as controls.

Cytotoxicity Assays. Spleen cell suspensions were added to the target cells in 250 μl of EMEM10, using replicates of four wells. The assay was incubated for 16 hr at 37° in 5% CO_2/95% air at 100% humidity. Plates were then centrifuged at $150 \times g$ for 10 min, and 150 μl of supernatant was removed from each well and assayed for radioactivity in a scintillation spectrophotometer.

Results were generally calculated as specific ^{51}Cr release (17), by the formula: (Ii − Ni)/(Wi − Ni), in which I = immune spleen cells, N = normal spleen cells, W = water lysis, and i = virus-infected target cells. Similar calculations were made for control target cells, but ^{51}Cr release in this case was always at background levels. The values given in Fig. 1 were not corrected for background, in order to show levels of absolute ^{51}Cr release which occur in this assay system.

Table 2. Cell-mediated lysis* is maximal for interactions sharing *H-2* genes

Mouse strain†	*H-2* type KD	MC57G (*bb*) 100:1	MC57G (*bb*) 50:1	MNB (*kd*) 100:1	MNB (*kd*) 50:1
B6	bb	43	29	14	7
B10.A (5R)	bd	29	16	72	30
B10.A	kd	12	18	87	50
A/J	kd	15	3	62	40
CBA/J	kk	10	10	46	25
BALB/c	dd	6	8	20	8

* In terms of percent specific ^{51}Cr release.
† Spleen cells from mice were assayed at 7 days after intraperitoneal inoculation with $10^{7.0}$ PFU of live ERA virus.

Immunosuppression. Mice were immunosuppressed by intraperitoneal inoculation with cyclophosphamide (Cytoxan, Mead Johnson, Ind.), 150 mg/kg, or hydrocortisone acetate (Wolins, Melville, N.Y.), 2.5 mg. In some experiments, mice were also passively immunized with 10 international units per mouse of antirabies serum (18).

RESULTS

Preliminary experiments (unpublished data) showed that, of several established cell lines tested, only MNB cells from A/J (*H-2*^{a}) mice and MC57G of C57BL/6 (B6, *H-2*^{b}) origin were suitable for use as targets for virus infection. In both lines, release of ^{51}Cr was substantially linear over the first 20 hr of assay. The results for the MNB line are shown in Fig. 1, expressed as total ^{51}Cr release (10) to illustrate the background levels of lysis occurring in this system. For simplicity of presentation, however, all subsequent data were calculated as specific ^{51}Cr release (17).

Maximal lymphocyte-mediated lysis was detected at 6 days after the immunization with live rabies virus (Fig. 2) and showed kinetics similar to those found in other virus systems (19, 20). Effector function was virus-specific (Table 1) and was always greater for interactions in which target and spleen populations shared *H-2* genes mapping at the *H-2K* or *H-2D* locus (Table 2). The levels of cross-reactivity between *H-2*-incompatible systems, however, were considerably greater than those found previously for other viruses (17).

A potent cell-mediated cytotoxic response was also generated

Table 1. Specificity* I of cytotoxic activity of spleen cells from rabies-immune or LCMV-immune mice

	Target cells: Rabies-infected		LCMV-infected	
Spleen/target cell ratio	100:1	50:1	100:1	50:1
Spleen cells from B6 mice:				
Rabies-immune†	37	24	0	0
LCMV-immune‡	7	2	43	24

* In terms of percent specific ^{51}Cr release from MC57G target cells.
† Injected intraperitoneally 7 days previously with $10^{7.0}$ PFU of live ERA virus.
‡ Injected intraperitoneally 8 days previously with $10^{3.3}$ median lethal doses (LD_{50}) of LCMV.

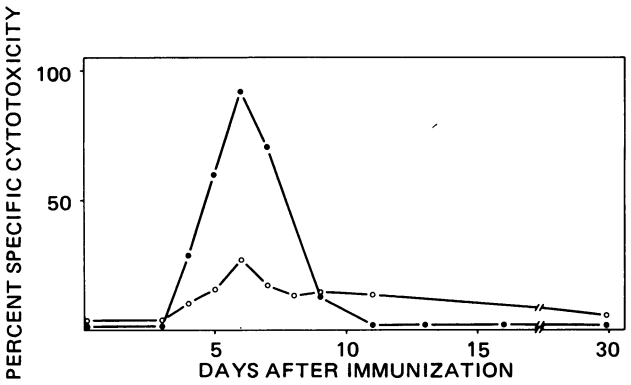

FIG. 3. Cytotoxicity of immune B6 (*H-2*^{b}) spleen cells for H-2 compatible (MC57G) (●) and *H-2* incompatible (MNB) (○) virus-infected target cells. Mice were immunized with 0.1 ml of undiluted, β-propiolactone-inactivated rabies vaccine.

after intraperitoneal inoculation of mice with β-propiolactone-inactivated rabies virus (Fig. 3). Specific ^{51}Cr release was maximal in the syngeneic targets, but considerable lysis also occurred in allogeneic, or *H-2*-incompatible, virus-infected targets. Depletion of cell-mediated cytotoxicity in both syngeneic and allogeneic targets, mediated by spleen cells from mice immunized with live or inactivated virus, was achieved by prior treatment of spleen cells with anti-thymus antiserum and complement (Table 3). Specific ^{51}Cr release, however, was not diminished by passage of lymphocyte populations through nylon-wool columns (Table 3), which remove most bone marrow-derived cells (13). Lysis of both *H-2* compatible and *H-2*-incompatible virus-infected cells would thus seem to reflect activity of virus-immune T cells. Furthermore, attempts to demonstrate antibody-dependent cell-mediated cytotoxicity (21, 22) in this system met with failure (Fig. 4).

Further characterization of the cell-mediated cytotoxic response in rabies infection revealed that, as for LCMV (19), cytotoxicity was completely abrogated by immunosuppression

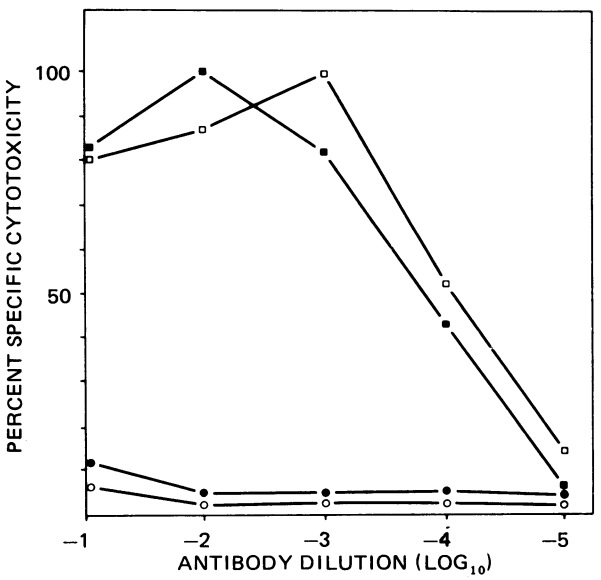

FIG. 4. Virus-infected MNB target cells were exposed for 1 hr to different dilutions of antirabies antibody (Ab) and then overlaid (100:1) with spleen cells from normal A/J mice in the presence or absence of guinea pig complement (C). The assays were incubated for 16 hr at 37°. ○, Ab alone; □, Ab + C; ●, Ab + spleen cells; ■, Ab + spleen cells + C.

of mice with cyclophosphamide (Table 4). Cortisone had no effect when administered on day 2 after immunization, but a considerable decrease in lytic activity was observed in two experiments when the drug was given on day 4 (Table 5 and unpublished data). The cell-mediated cytotoxic response of mice to live virus was totally prevented by prior inoculation with specific antibody (Table 6), though mice still generated cytotoxic T cells when injected with the β-propiolactone-inactivated vaccine (Table 6). This probably reflects the higher concentration of antigen in the vaccine (8) than in live virus preparation (Fig. 5). The events necessary for T cell stimulation are

Table 3. Characterization* of lymphocyte populations by treatment with anti-thymus serum or passage through nylon-wool columns

Mouse strain	Immunogen†	Target cells	Treatment‡	Spleen/target cell ratio 100:1	50:1	25:1
A/J (*H-2*^{a})	Virus	MNB (*H-2*^{a})	Anti-Thy-1+c	1	3	0
			C	70	25	13
			Nil	62	40	26
B6 (*H-2*^{b})	Virus	MNB (*H-2*^{a})	Anti-Thy-1+c	1	0	0
			C	13	8	3
			Nil	15	3	0
B6 (*H-2*^{b})	Vaccine	MC57G (*H-2*^{b})	Anti-Thy-1+c	8	4	0
			C	48	15	0
			Nil	60	28	3
B6 (*H-2*^{b})	Vaccine	MNB (*H-2*^{a})	Anti-Thy-1+c	0	0	0
			C	20	8	0
			Nil	35	9	3
B10.A (*H-2*^{a})	Virus	MNB (*H-2*^{a})	Nylon-wool	41	45	15
			Nil	43	34	15
B10.A (*H-2*^{a})	Virus	MC57G (*H-2*^{b})	Nylon-wool	16	8	7
			Nil	0	0	0

* In terms of percent specific ^{51}Cr release.

† Mice were injected intraperitoneally 7 days previously with either $10^{7.0}$ PFU of live ERA virus or 0.1 ml of rabies vaccine.

‡ Spleen populations were treated with either anti-thymus serum and guinea pig complement (Anti-Thy-1+c) (12) or by passage through nylon-wool columns (13) or were untreated (Nil).

Table 4. Immunosuppressive effect* of cyclophosphamide

Day treated†	Spleen /target cell ratio 100:1	50:1	25:1
−2	6	0	0
+1	0	0	0
Control	93	54	26

* In terms of percent specific ^{51}Cr release from MNB target cells.
† A/J mice were injected intraperitoneally with cyclophosphamide, 150 mg/kg at 2 days before or 1 day after intraperitoneal inoculation with $10^{7.0}$ PFU of live rabies virus (day 1). Spleen populations were assayed 7 days after exposure to virus.

apparently established by 24 hr after immunization, because antibody given at this time had little effect (Table 6).

DISCUSSION

Virus-immune T cells generated in mice inoculated with live or inactivated rabies virus showed, in some experiments, substantial cytotoxicity for *H-2*-incompatible virus-infected target cells. This is contrary to the situation found for LCMV, the poxviruses, paramyxoviruses, and myxoviruses (ref. 17 and unpublished data), with which lytic interactions are only recognized when T cell and target cell share genes mapping at *H-2K* or *H-2D*. This discrepancy, however, should not be given undue emphasis. Release of ^{51}Cr from rabies virus-infected cells was always maximal in the syngeneic situation. Furthermore, the experiments with rabies were all performed with two established tumor cell lines; we have not yet found primary cell cultures that function satisfactorily as targets. Most of the *H-2* genetic mapping studies of other viruses has been done with mouse peritoneal macrophages as target cells (23, 24). Specific cell-mediated lysis of trinitrophenyl-modified lymphocytes also requires a sharing of *H-2* genes, but tumor cells used in this assay system also have shown cell-mediated cytotoxicity in *H-2*-incompatible situations (25).

Another apparent anomaly in the rabies system is that, unlike the situation for LCMV (19), late treatment (i.e., 4 days after infection) with cortisone diminishes the T cell response. Perhaps this indicates that the cortisone is removing a stimulator cell population required for continuing lymphocyte proliferation (26), rather than directly damaging the T cells themselves. Treatment with cortisone 2 days after antigen inoculation may allow recovery of such cells or reprocessing of the antigen. The fact that this situation differs from that with LCMV may reflect

Table 5. Diminution* of cell-mediated cytotoxicity in mice by immunosuppression with cortisone

Day treated†	Target cells: MC57G (B6) B6‡	MC57G (B6) B10.A‡	MNB (A/J) B6	MNB (A/J) B10.A
+2	80	4	26	87
+4	35	0	0	38
Control	90	4	16	83

* In terms of percent specific ^{51}Cr release.
† Intraperitoneal injection of hydrocortisone acetate, 2.5 mg, at 2 or 4 days after immunization.
‡ Mice were immunized with a single intraperitoneal injection of 0.1 ml of rabies vaccine (lot 2636/11) 7 days previously, and spleen cells were assayed at a ratio of 100:1.

Table 6. Effect* of antibody treatment on cell-mediated cytotoxic response

Day treated†	Treatment‡ Live ERA	Vaccine
−1	1	35
+1	48	79
Control	67	70

* In terms of percent specific ^{51}Cr release from MC57G targets.
† Injected intraperitoneally with 10 international units of rabies antibody at 1 day before or 1 day after immunization.
‡ Immune spleen cells were assayed (100:1) at 7 days after injection with $10^{7.0}$ PFU of live ERA virus or with 0.1 ml of rabies vaccine.

the fact that the rabies virus multiplies exclusively in nerve cells, whereas LCMV replicates to high titers in many tissues. Even in the LCMV model, however, administration of cortisone 3 or 5 days prior to the processing of spleen cells enriches the population of cytotoxic T cells, whereas treatment 24 hr before sampling results in no increase in T cell activity (19).

Possibly the most important finding from these experiments is that immunization with a β-propiolactone-inactivated virus preparation generates a strong cytotoxic T cell response. Furthermore, this concentrated vaccine (8) is apparently more immunogenic than is live virus. This has not been shown previously in other virus systems, perhaps because insufficient concentrations of antigen were used. A similar inactivated rabies vaccine produced from human diploid cell cultures does not cause adverse side-effects in man (27, 28). Field studies indicate that, insofar as protection against rabies is concerned (29), it is an extremely effective immunogen.

Treatment of animals with rabies immune serum before the administration of antigen effectively blocks the generation of specific immune T cells, just as it blocks the formation of virus-neutralizing antibody (30).

Our immediate experimental task is to elucidate the cellular basis of vaccine treatment. This should be feasible now that an *in vitro* T cell assay is available. Hopefully, this may allow development of optimal vaccination schedules for use in people exposed to rabies. The preliminary findings reported here in-

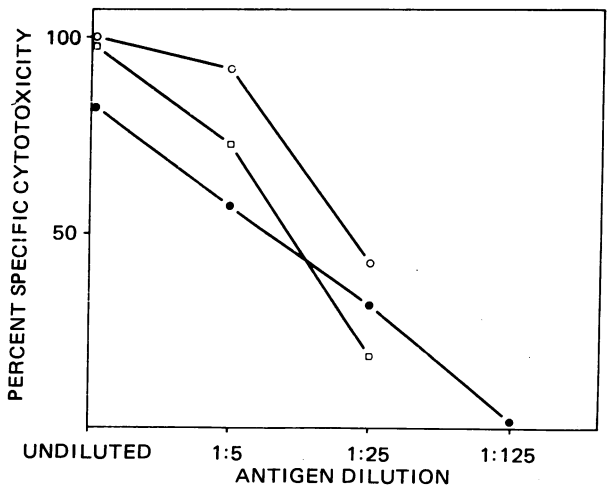

FIG. 5. Dose-dependence of the cell-mediated cytotoxic response after immunization with fivefold dilutions of live (●) or β-propiolactone inactivated (○, concentrated; □, purified) ERA virus preparations. Lymphocyte populations from A/J mice were taken 7 days after primary immunization and overlaid on virus-infected *H-2*-compatible MNB target cells at a ratio of 100:1.

dicate that interference between passive antibody and active induction of antibody by vaccine could be prevented if the rabies antiserum is administered some time after vaccine inoculation and not simultaneously as is generally practiced (31). Adoption of such protocols depends, however, on demonstration that the T cell response in rabies is protective. At present there is only indirect evidence to support this viewpoint.

We thank Slawa Kosiewicz and Linnea R. Clompus for excellent technical assistance. This investigation was supported in part by USPHS Research Grants AI 09706 from the National Institute of Allergy and Infectious Diseases, NS 11036 from the National Institute of Neurological Diseases and Stroke, and RR 05540 from the Division of Research Resources and by funds from the World Health Organization.

1. Turner, G. S. (1973) *Nature New Biol.* **241**, 90–92.
2. Kaplan, M. M., Wiktor, T. J. & Koprowski, H. (1975) *J. Immunol.* **114**, 1761–1765.
3. Wiktor, T. J., Kamo, I. & Koprowski, H. (1974) *J. Immunol.* **112**, 2013–2019.
4. Pasteur, L. (1885) *C.R. Hebd. Seances Acad. Sci.* **101**, 765–774.
5. Anderson, J. A., Daly, F. T. & Kidd, J. C. (1966) *Ann. Intern. Med.* **64**, 1297–1302.
6. Hattwick, M. A. W., Hochberg, F. H., Landrigan, P. J. & Gregg, M. B. (1972) *J. Am. Med. Assoc.* **222**, 44–47.
7. Abselseth, M. K. (1967) Dissertation, University of Minnesota, St. Paul.
8. Wiktor, T. J., Sokol, F., Kuwert, E. & Koprowski, H. (1969) *Proc. Soc. Exp. Biol. Med.* **131**, 799–805.
9. World Health Organization. (1973) *Laboratory Techniques in Rabies* (Monograph Series no. 23), 3rd ed.
10. Zinkernagel, R. M. & Doherty, P. C. (1974) *Scand. J. Immunol.* **3**, 287–294.
11. Boyle, W. (1968) *Transplantation* **6**, 761–764.
12. Doherty, P. C., Zinkernagel, R. M. & Ramshaw, I. A. (1974) *J. Immunol.* **112**, 1548–1552.
13. Julius, M. H., Simpson, E. & Herzenberg, L. A. (1973) *Eur. J. Immunol.* **3**, 645–649.
14. Miller, C. A. & Levine, E. M. (1974) *J. Neurochemistry* **22**, 751–758.
15. Aden, D. P. & Knowles, B. B. (1976) *Immunogenetics* **3**, 209–221.
16. Wiktor, T. J., Kuwert, E. & Koprowski, H. (1968) *J. Immunol.* **101**, 1271–1282.
17. Doherty, P. C. & Zinkernagel, R. M. (1976) *Immunology* **31**, 27–32.
18. Schlumberger, H. D., Wiktor, T. J. & Koprowski, H. (1970) *J. Immunol.* **105**, 291–298.
19. Doherty, P. C. & Zinkernagel, R. M. (1974) *Transplant. Rev.* **19**, 89–120.
20. Doherty, P. C., Blanden, R. V. & Zinkernagel, R. M. (1976) *Transplant. Rev.* **29**, 89–124.
21. Perlmann, P., Perlmann, H. & Wigzell, H. (1972) *Transplant. Rev.* **13**, 91–114.
22. MacLennan, I. C. M. (1972) *Transplant. Rev.* **13**, 67–90.
23. Zinkernagel, R. M. & Doherty, P. C. (1975) *J. Immunol. Methods* **8**, 263–266.
24. Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. & David, C. S. (1975) *Nature* **254**, 269–270.
25. Shearer, G. M., Rehn, T. G. & Schmitt-Verhulst, A.-M. (1976) *Transplant. Rev.* **29**, 223–248.
26. Zinkernagel, R. M. & Doherty, P. C. (1975) *J. Exp. Med.* **141**, 1427–1436.
27. Wiktor, T. J., Plotkin, S. A. & Grella, D. W. (1973) *J. Am. Med. Assoc.* **224**, 1170–1171.
28. Plotkin, S. A., Wiktor, T. J., Koprowski, H., Rosanoff, B. I. & Tint, H. (1976) *Am. J. Epidemiol.* **103**, 75–80.
29. Bahmanyar, M., Fayaz, A., Nour-Salehi, S., Mohammadi, M. & Koprowski, H. (1976) *J. Am. Med. Assoc.*, in press.
30. Wiktor, T. J., Lerner, R. A. & Koprowski, H. (1971) *Bull. WHO* **45**, 747–753.
31. WHO Expert Committee on Rabies (1973) *Sixth Report* (World Health Organization, Technical Reports Series), p. 523.