

**Splenocytes of Mice with Induced Immunological Tolerance
to Plant Antigens for Construction
of Hybridomas Secreting Tomato Spotted Wilt Virus-Specific Antibodies**

H. T. Hsu, Y. C. Wang, R. H. Lawson, M. Wang, and D. Gonsalves

First three authors: Research microbiologist, visiting scientist, and research plant pathologist, United States Department of Agriculture, Agricultural Research Service, Florist and Nursery Crops Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705; fourth and fifth authors: graduate research assistant and professor, Cornell University–New York State Agricultural Experiment Station, Department of Plant Pathology, Geneva, NY 14456.

The second author was on leave of sabbatical studies from the Chinese Academy of Agricultural Sciences, Biotechnology Research Center, Beijing, China.

Cooperative investigation, U. S. Department of Agriculture, Agricultural Research Service, BARC-West and Cornell University–New York State Agricultural Experiment Station, Geneva. Portion of the research is part of a thesis to be submitted by the fourth author in partial fulfillment for the Ph.D. degree.

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply approval to the exclusive of other products that may also be suitable.

We gratefully acknowledge L. M. Black, E. L. Civerolo, R. H. Converse, G. Loebenstein, and M. R. McLaughlin for critical review of the manuscript and valuable suggestions.

Accepted for publication 15 August 1989 (submitted for electronic processing).

ABSTRACT

Hsu, H. T., Wang, Y. C., Lawson, R. H., Wang, M., and Gonsalves, D. 1990. Splenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus-specific antibodies. *Phytopathology* 80:158-162.

Immune tolerance or suppression of immune response to plant antigens was successfully induced by injecting neonatal BALB/c mice four times with healthy plant extracts on days 1, 3, 5, and 7 after birth. Subsequently, two mice each were injected once with partially purified tomato spotted wilt virus (TSWV) at age 5, 7, or 9 wk, respectively. Splenectomy and fusion of spleen cells with P3/NS1/1-Ag-4-1 myeloma cells were performed 4 days after TSWV immunization for each group. Fusion products were evaluated by ELISA on plates coated with TSWV and on plates on which TSWV was trapped by adsorbed virus-specific rabbit polyclonal antiserum. Percentages of TSWV-specific hybridomas were 83, 50, and 40% for groups of mice immunized at 5, 7, and 9 wk of age, respectively.

For control mice that did not receive neonatal injections of normal host antigens, the TSWV-specific hybridomas were 0, 7, and 7%, when immunized at 5, 7, and 9 wk, respectively. Conventional immunization of adult mice over a period of time with multiple injections of immunogens resulted in about 3–12% of hybridomas being TSWV-specific. The selection of virus-specific hybridomas may best be achieved when mice, which have been rendered immunologically tolerant to normal host antigens, are immunized at the age of 5 wk. This method is useful in the generation of hybridomas for antigens that are difficult to purify or concentrate and in the preparation of hybridomas to minor distinct epitopes that are difficult to separate.

Additional keywords: immunosuppression, induced-tolerance, monoclonal antibody, tolerogenic, tolerogen.

Tomato spotted wilt virus (TSWV) is vectored by thrips (13). It infects a wide range of plant species in many families (2,8,13). TSWV-infected plants may not show severe symptoms until the

onset of flowering or following a period of stress. TSWV antigen may be detected serologically in diseased plants (8,15), but disease diagnosis is restricted by the lack of availability of high quality antisera (13). Purified TSWV is unstable and rapidly breaks down once outside the tissue of its hosts (5). It is difficult to prepare sufficient amounts of purified virus suitable for immunization. Many procedures for purifying TSWV have been reported

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1990.

(5,13,15). Difficulties in successfully repeating in one laboratory a published procedure for purifying TSWV developed in others may reflect different intrinsic properties of the virus isolate in each study. The specific transmission of TSWV isolates (31,32) by different thrips species may furnish a basis for differentiation and classification of strains (3,26,35). One of the objectives of this study is to develop discriminating monoclonal antibodies to differentiate strains of TSWV.

Suitable immunogens for TSWV (1) were first prepared more than 30 yr ago by using a mild extraction method (4) and a density gradient centrifugation procedure (6). Although the titers of the antisera were low, they reacted specifically to intact TSWV and soluble antigens in subviral preparations in ring precipitin tests. It was also shown that the subviral preparation contained a higher concentration of specific antigens than did the purified virus preparation (1; Hsu and Lawson, *unpublished data*). Subsequent attempts to produce TSWV antisera were made by other investigators (13). Only recently, by a procedure described by Black et al (5) modified by Mohamed et al (29) to purify the virus, an improved antiserum was prepared for a strain of TSWV (15). This antiserum has been used to detect TSWV in numerous plants (8,15) and in thrips (9).

Techniques for hybridization of myeloma cells with spleen cells secreting antibody permit the production of unlimited amounts of antibodies from immortal hybridoma cell lines (25). It is difficult to select hybridomas for those antigens that cannot be extensively purified, because of the high proportion of non-target antibodies and non-target antigens present. Despite such problems, murine monoclonal antibodies have been produced to numerous plant viruses that are poorly immunogenic in animals and/or are difficult to purify, including TSWV (33).

Enhancement of the proportion of spleen cells secreting antibodies to target antigens prior to cell hybridization is possible. A method for enrichment of specific antibody-producing splenocytes prior to cell fusion has been reported for cauliflower mosaic virus (14). We have taken a different approach to achieve the enhancement of specific antibody-producing hybridomas. Immunological tolerance is an immune response that can be induced by injecting excess amounts of an antigen into animals, and is characterized by the absence of detectable antibodies following the first injection and subsequent challenge injections (10,12). An antigen that is capable of inducing a state of immunological tolerance is "tolerogenic." In the present paper, we describe a method of enhancing the proportion of TSWV-specific antibody secreting hybridomas by suppressing development of non-target antibodies to host plant constituents in mice. A preliminary report has been published (24).

MATERIALS AND METHODS

Viruses. Isolates of TSWV were maintained in *Datura stramonium* L. at Geneva, NY. An isolate from Oklahoma (TSWV-OK from *Nicotiana glutinosa* L.) from John L. Sherwood of Oklahoma State University and an isolate from Hawaii (TSWV-BL from Batavia lettuce, *Lactuca sativa* L.) (15) were used in the present study.

Preparation of viral antigens. Viral antigens were prepared at Cornell University, Geneva, NY, according to a procedure previously described (15). Leaf tissues of *D. stramonium* infected by TSWV were ground in potassium phosphate and sodium sulfite buffer. The extract was centrifuged at 10,000 g for 15 min. Pellets were resuspended in 0.01 M sodium sulfite and the suspension was subjected to differential centrifugation. Virus preparations were further centrifuged in a sucrose density gradient. The virus zones were collected and concentrated by centrifugation at 100,000 g for 60 min. Final virus preparations were resuspended in 0.01 M sodium sulfite and sent, under a permit (permit No. 1404 issued by the U. S. Department of Agriculture, Animal and Plant Health Inspection Service) from Geneva, NY, to Beltsville, MD. A total of 24 OD units (A_{254}) of TSWV-BL and 18 OD (A_{254}) units of TSWV-OK were shipped. Virus preparations were adjusted to

1.0 OD unit (A_{254}) per 200 μ l and were stored frozen at -70°C .

Preparation of plant antigens (tolerogens). Healthy *D. stramonium* antigens were prepared by the same procedure used for viral antigens (15). After sucrose density gradient centrifugation in a Beckman SW28 rotor, materials located between 3–6 cm from the bottom of tubes were collected and pelleted by centrifugation at 300,000 g for 60 min in a Beckman Type 50.2 Ti rotor at 4 C. The resulting pellets were resuspended in phosphate-buffered saline (PBS, 0.02 M phosphate, 0.15 M NaCl, pH 7.4) to give a 50-fold concentration of plant antigens (i.e., 50 g of tissues resulted in 1 ml of plant antigen preparation in PBS). The resuspended pellet was stored in 100- μ l aliquots at -70°C until use.

Mice. Pregnant and 20-g female virgin adult BALB/c mice were obtained from Dominion Laboratories (Dublin, VA). Pregnant mice were observed daily for newborns. Gloves were worn for handling newborns and 26 G or 27 G needles were used for injecting newborns.

Injections. Six newborn mice from the same litter were each injected intraperitoneally with 25, 40, 100, and 200 μ l of healthy *D. stramonium* antigens (tolerogens) on days 1, 3, 5, and 7, respectively. Six sibling mice from a second litter did not receive healthy plant antigen, tolerogen, injections. These were used as controls. Two mice at 5 wk, 2 at 7, and 2 at 9 wk for each group were immunized intraperitoneally with a single injection of 200 μ l of a TSWV preparation when the mice were 5, 7, or 9 wk old. Splenectomies were performed 4 days after the immunization, and fusions were made immediately.

For immunization of adult mice, a conventional injection schedule was employed as previously reported (21). Six 20 g-female mice were injected 3 to 4 times with 200 μ l each of TSWV preparations per injection. The first injection with immunogen in complete Freund's adjuvant, and the second injection with immunogen in incomplete Freund's adjuvant (IFA), were 4 wk apart. Four of the six adult mice received a final injection of 200 μ l each of TSWV 1 wk after the second injection. The two remaining mice received four immunizations, with a third injection (with IFA) given 4 wk after the second injection, and the final injection 1 wk later. Mice in all treatment groups were sacrificed 4 days after the final injection, and spleen cells were prepared for hybridization.

Myelomas and culture media. The myeloma cell line P3/NS1/1-Ag4-1 used in previous studies (21) was maintained in a complete medium containing RPMI-1640 medium (Gibco Laboratories, Grand Island, NY), 1 mM L-glutamine, 1 mM pyruvate, and 5.7% of each of Nu-serum (Collaborative Research, Inc., Bedford, MA), CPSR-3 controlled process serum replacement (Sigma Chemical, Inc., St. Louis, MO) and HyClone fetal bovine serum, defined (HyClone Laboratory, Inc., Logan, UT).

Fusion and selection medium. The preparation of myeloma cells, single splenocyte suspensions, and methods of fusion were similar to those previously described (21). After fusion, cells derived from mice that received a single immunization of TSWV were resuspended in 120 ml of HAT selection medium (27). Those from mice that received multiple immunizations were resuspended in 250 ml of HAT medium because of larger spleens. The HAT selection medium consisted of 9 parts complete medium and 1 part myeloma cell-conditioned complete medium and was supplemented with 10^{-1} mM hypoxanthine, 4×10^{-4} mM aminopterin, 1.6×10^{-2} mM thymidine, 6 mM Hepes, and 2×10^{-2} mM 2-mercaptoethanol. Fused cells were incubated in T-75 culture flasks in a 6–7% CO_2 atmosphere at 36 C in a humidified incubator. A 2- to 3-hr incubation without disturbance before plating in 96-well culture plates was sufficient to prevent the growth of fibroblasts in cultures. After a gentle rocking motion of the flask, cell suspensions were transferred to a petri dish and were seeded with 0.2 ml/well in 96-well culture plates, and incubated in CO_2 incubators. Twice a week, starting 4 days after fusion, 100 μ l of medium was replenished by fresh HAT medium. Eleven days after fusion, aminopterin was omitted from the HAT medium.

RESULTS

ELISA. Two types of indirect ELISA were used to identify hybridoma cultures secreting antibodies specific to TSWV or normal host antigens. Before adding hybridoma culture supernatants, TSWV antigens were trapped on polyvinyl chloride (PVC) plates either by: A) direct coating at pH 9.6 in 0.1 M carbonate buffer (23) or B) incubating a TSWV preparation in PVC plates coated with rabbit anti-TSWV serum at pH 7.4 in PBS at room temperature for 2 hr (21). Rabbit anti-TSWV sera were prepared at Cornell University (15). The use of either one of the prepared ELISA plates alone may affect the selection of antibodies suitable only in one type of ELISA but not the other (22). For controls, concentrated extracts from healthy *D. stramonium* were used instead of the virus antigens. After a 30-min blocking incubation with 1% bovine serum albumin, alkaline phosphatase-labeled goat anti-mouse IgA, IgG, and IgM immunoglobulins (Kirkgaard and Perry Laboratory, Gaithersburg, MD) were used to detect the presence of mouse antibodies. Hybridoma culture fluids from each well were collected twice at two different times and were tested separately.

Immunoglobulin class determination. Tomato spotted wilt virus antibody immunoglobulin classes and subclasses were identified in Ouchterlony double diffusion tests as described (21). Rabbit antisera to mouse IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM, and homologous antigens, were purchased from Litton Bionetics (Charleston, SC).

In mice injected first with normal host antigens (tolerogens) followed by the viral antigens, percentages of TSWV-specific hybridomas were considerably greater (Table 1) than in those mice of the same age that received only the viral antigen (Table 2). Although the lowest number of virus-specific hybridomas was derived from mice immunized first with healthy plant antigens when they were 5 wk old, the percentage of virus-specific hybridomas was higher in these samples than it was among animals 7 or 9 wk old when injected with viral antigen (Table 1). The average percentages of TSWV-specific hybridomas derived from 5-, 7-, and 9-wk-old tolerance-induced mice were 83, 50, and 40% respectively. The percentages of TSWV-specific hybridomas derived from tolerance-induced mice were comparable for both strains of TSWV when tested at 5 and 9 wk (Table 1). We were not able to collect data for the TSWV-OK strain for the 7-wk-old mouse injected with healthy plant antigens because of spleen cell loss.

Fewer TSWV-specific hybridomas were generated from control mice that were not preimmunized with healthy plant antigens. No TSWV-specific hybridomas were produced from 5-wk-old mice that were not immunized first with plant antigens (Table 2). On the other hand, numbers of hybridoma antibodies reactive to plant antigens were fivefold greater than those obtained for

TABLE 1. Specificities (number and percentage) of hybridomas derived from tomato spotted wilt virus (TSWV) immune splenocytes of mice neonatally injected with plant constituents followed by injection with strain BL or strain OK at the age of 5, 7, or 9 wk^a

Age of mice when immunized with TSWV	Hybridoma specificities								Percentage of virus specific hybridomas
	BL-immunization				OK-immunization				
	Virus specific		Host specific		Virus specific		Host specific		
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	
5	6	86	1	14	9	82	2	18	83
7	11	50	11	50	... ^b ^b	...	50
9	30	41	44	59	23	39	36	61	40

^aNewborn mice were injected intraperitoneally with 25, 40, 100, and 200 μ l of partially purified healthy *Datura stramonium* antigens, respectively, on day 1, 3, 5, or 7. A single immunization of 200 μ l of TSWV-BL or TSWV-OK preparation was followed when mice were 5, 7, and 9 wk old. Splenectomies were performed 4 days after immunization for each age group.

^bLoss of cells occurred during the procedure.

TABLE 2. Specificities (number and percentage) of hybridomas from pairs of mice, each pair receiving a single immunization of tomato spotted wilt virus (TSWV-BL or TSWV-OK) when 5, 7, or 9 wk old^a

Age of mice when immunized with TSWV	Hybridoma specificities								Percentage of virus specific hybridomas
	BL-immunization				OK-immunization				
	Virus specific		Host specific		Virus specific		Host specific		
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	
5	0	0	9	100	0	0	7	100	0
7	1	6	17	94	2	9	21	91	7
9	5	8	54	92	4	6	59	94	7

^aSix sibling mice from the same litter were immunized with 200 μ l of TSWV-BL or TSWV-OK preparation at the age of 5, 7, or 9 wk. Splenectomies were performed 4 days after immunization for each age group. Note, these are standard controls for the tolerogen-induced mice in Table 1.

TABLE 3. Specificities (number and percentage) of hybridomas derived from mice that received multiple immunizations of tomato spotted wilt virus (TSWV-BL or TSWV-OK)^a

Experiments	Hybridoma specificities							
	BL-immunization				OK-immunization			
	Virus specific		Host specific		Virus specific		Host specific	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
I	5	3	135	97	3	4	68	96
II	7	8	81	92	9	12	65	88
III	5	3	186	97	7	6	103	94

^aAll adult mice were obtained from a supplier. Mice were immunized 3 times with either TSWV-BL or TSWV-OK preparation in experiments I and II, and 4 times in experiment III. Splenectomies were performed 4 days after the last immunization. Note, these are conventional procedures used for production of hybridomas.

mice of the same age first injected with healthy plant antigens (Table 1 and 2). Between 5 and 10% of the hybridomas produced in mice immunized at 7 or 9 wk with TSWV preparations were virus specific (Table 2). The number of hybridomas secreting antibodies specific to TSWV-OK, TSWV-BL, or non-target host plant antigens was greater from mice immunized at 9 wk than from mice immunized at 5 wk (Tables 1 and 2).

Hybridomas secreting TSWV-specific antibodies varied from 3 to 9 per spleen/fusion for those mice that only received multiple immunizations of TSWV (Table 3). Multiple immunizations produced a larger number of plant antigen-specific hybridomas ranging from 65 per spleen/fusion to as many as 185 per spleen/fusion (Table 3) than for a single immunization (Table 1 and 2).

The highest percentage of TSWV hybridoma antibodies produced from mice immunized only once with viral preparations was in the IgM class (Table 4). The next most frequently secreted antibody type by hybridomas derived from single immunizations was IgG1. Neither IgG2b nor IgG3 was detected in hybridomas derived from spleens of mice immunized only once. Following multiple immunization with TSWV preparations, the IgG1 subclass was the most commonly produced type of virus-specific antibody (Table 4). Less than 3% of antibodies were IgM.

DISCUSSION

Immunological tolerance to plant antigens was induced in mice by injecting newborn mice with plant extracts. Induction of immunological unresponsiveness in adult animals following injection neonatally with a tolerizing antigen has been previously described (17,19,34). Unlike immunosuppression, which is nonspecific, immunological tolerance is a form of specific responsiveness in which the animal's immune system responds in a negative way to a specific antigenic stimulation (18). Tolerance of an immunogen is revealed by the absence of synthesis of the corresponding antibodies and can be induced, newborn and adult, in chickens, mice, or rabbits with a variety of conventional immunogens such as serum proteins and bacterial polysaccharides (10-12,17,19,34). Tolerance is more readily established in the newborn than in adult animals (7,10,28,30,36). Induction of tolerance to plant antigens in mice before immunization with plant virus preparations offers a significant improvement over the standard methods of immunization in producing hybridomas to TSWV and possibly other plant viruses (Table 1 and 3).

The immune system of the mouse was still being developed in 5-wk-old mice. As a consequence, there were increasing numbers of immunogen-specific (both TSWV and plant antigens) hybridomas derived from mice of increasing ages (Table 2). Development of the immune system also resulted in increasing numbers of TSWV-specific hybridomas in mice rendered immunologically tolerant to plant antigens in relation to the increasing age of mice at the time of immunization (Table 1). Although not completely achieved in these experiments, a higher degree

TABLE 4. Immunoglobulin classes and subclasses of tomato spotted wilt virus antibodies secreted by hybridomas derived from spleens of mice that received single or multiple immunizations^a

Immunoglobulin class/subclass	Single immunization		Multiple immunizations	
	Number	Percentage	Number	Percentage
IgA	0	0	0	0
IgG1	14	18	27	75
IgG2a	1	1	5	14
IgG2b	0	0	2	5
IgG3	0	0	0	0
IgM	61	77	1	3
Others ^b	3	4	1	3
Total	79	100	36	100

^a Figures in single immunizations represent a total of five fusions from mice rendered tolerant to healthy plant antigens in Table 1; those in multiple immunizations represent a total of six fusions in Table 3.

^b Could not be isotyped.

of tolerance was induced in 5-wk-old mice than in 7- or 9-wk-old mice and was successfully used in obtaining immune splenocytes for target antigens.

Complete tolerance to plant antigens was not demonstrated in these studies (Table 1). This failure to induce complete tolerance is probably due to the heterogeneous nature of constituents in the plant extract (tolerogen preparations) in which some components may be present in suboptimal concentrations for induction of tolerance. It is also possible that antigens in low concentration in healthy plants may be elevated in concentration after infection. A diminishing tolerance state of these mice with increasing ages may also account for the increase in the number of plant antigen-specific hybridomas (Table 1). It is unlikely that plant constituents of highly variable composition will induce a degree of tolerance comparable to those attained by homogeneous tolerogens (10,12). We did not, however, attempt to analyze the specific plant antigens that conferred tolerance.

The majority of the hybridoma antibodies derived from tolerance-induced mice receiving a single immunization were IgM, whereas more than 90% of the antibodies secreted by hybridomas derived from mice that received multiple injections of immunogens were IgG. Immunoglobulin M antibodies are serologically difficult to handle (16). They are generally of relatively low affinity and rather resistant to proteolytic fragmentation. However, IgM is very effective in complement-mediated cell lysis. Selection of IgG (IgG1, IgG2a, IgG2b, and IgG3) only antibodies can be easily achieved by using anti-mouse IgG specific immunoglobulin-enzyme conjugates in the screening procedure. Although not tested, procedures employing multiple immunizations of tolerance-induced mice that may increase proportion of IgG antibodies should not be overlooked.

Immunological tolerance established in the neonatal period provides an extremely useful approach for the production of target specific hybridomas for antigens that are difficult to obtain in pure form. This procedure also makes it possible to produce hybridomas that will distinguish very closely related antigens (20). The potential application of induced immunological tolerance in producing polyclonal antisera for plant viruses in rabbits would be of great interest to plant pathologists.

LITERATURE CITED

- Alden, E. 1956. The production of an antiserum against an antigen from an unstable plant virus. M. S. thesis. University of Illinois, 37 pp.
- Best, R. J. 1968. Tomato spotted wilt virus. *Adv. Virus Res.* 13:65-146.
- Black, L. M. 1944. Some viruses transmitted by agallian leafhoppers. *Proc. Am. Philos. Soc.* 88:132-144.
- Black, L. M., and Brakke, M. K. 1954. Serological reactions of a plant virus transmitted by leafhoppers. (Abstr.) *Phytopathology* 44:482.
- Black, L. M., Brakke, M. K., and Vatter, A. E. 1963. Purification and electron microscopy of tomato spotted wilt virus. *Virology* 20:120-130.
- Brakke, M. K. 1951. Density gradient centrifugation: a new separation technique. *J. Am. Chem. Soc.* 73:1847-1848.
- Cambier, J. C., Vitetta, E. S., Uhr, J. W., and Kettman, J. R. 1977. B cell tolerance. II. Trinitrophenyl human gamma globulin-induced tolerance in adult and neonatal murine B cells responsive to thymus-dependent and independent forms of the same hapten. *J. Exp. Med.* 145:778-787.
- Cho, J. J., Mau, R. F. L., Gonsalves, D., and Mitchell, W. C. 1986. Reservoir weed hosts of tomato spotted wilt virus. *Plant Dis.* 70:1014-1017.
- Cho, J. J., Mau, R. F. L., Hamasaki, R. T., and Gonsalves, D. 1988. Detection of tomato spotted wilt virus in individual thrips by enzyme-linked immunosorbent assay. *Phytopathology* 78:1348-1352.
- Dixon, F. J., and Maurer, P. H. 1955. Immunological unresponsiveness induced by protein antigens. *J. Exp. Med.* 101:245-257.
- Dresser, D. W. 1962. Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen. *Immunology* 5:378-388.
- Felton, L. D., and Ottinger, B. 1942. Polysaccharide as a paralyzing agent on the mechanism of immunity in white mice. (Abstr.) *J.*

- Bacteriol. 43:94-95.
13. Francki, R. I. B., and Hatta, T. 1981. Tomato spotted wilt virus. Pages 491-512 in: *Handbook of Plant Virus Infections and Comparative Diagnosis*. E. Kurstak, ed. Elsevier-North Holland Biomedical Press, New York.
 14. George, R. A., and Converse, R. H. 1988. Methods for the enrichment of desired B-cell populations before anti-cauliflower mosaic virus hybridoma formation. *Phytopathology* 78:1631-1636.
 15. Gonsalves, D., and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. *Plant Dis.* 70:501-506.
 16. Gooding, J. W. 1983. *Monoclonal Antibodies: Principles and Practice*. Academic Press Inc., New York. 276 pp.
 17. Hanan, R., and Oyama, J. 1954. Inhibition of antibody formation in mature rabbits by contact with the antigen at the early age. *J. Immunol.* 73:49-53.
 18. Herbert, W. J., and Wilkinson, P. C. 1971. *A Dictionary of Immunology*. Blackwell Scientific Publications, Oxford and Edinburgh. 195 pp.
 19. Hirata, A. A., Garvey, J. S., and Campbell, D. H. 1960. Retention of antigen in tissue of serologically suppressed chickens. *J. Immunol.* 84:576-581.
 20. Hockfield, S. 1987. A Mab to a unique cerebellar neuron generated by immunosuppression and rapid immunization. *Science* 237:67-70.
 21. Hsu, H. T., Aebig, J., and Rochow, W. F. 1984. Differences among monoclonal antibodies to barley yellow dwarf viruses. *Phytopathology* 74:600-605.
 22. Hsu, H. T., Franssen, J. M., van der Hulst, C. T. C., Derks, A. F. L. M., and Lawson, R. H. 1988. Factors affecting selection of epitope specificity of monoclonal antibodies to tulip breaking potyvirus. *Phytopathology* 78:1337-1340.
 23. Hsu, H. T., and Lawson, R. H. 1985. Comparison of mouse monoclonal antibodies and polyclonal antibodies of chicken egg yolk and rabbit for assay of carnation etched ring virus. *Phytopathology* 75:778-783.
 24. Hsu, H. T., Wang, Y. C., and Lawson, R. H. 1988. Induction of immunological tolerance to enhance the production of virus-specific hybridoma. (Abstr.) *Phytopathology* 78:1537.
 25. Kohler, G., and Milstein, C. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* 256:495-497.
 26. Kunkel, L. O. 1935. Possibilities in plant virus classification. *Bot. Rev.* 1:1-17.
 27. Littlefield, J. W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* 145:709-710.
 28. Metcalf, E. S., Schrater, F., and Klinman, N. R. 1979. Murine models of tolerance induction in developing and mature B cells. *Immunol. Rev.* 43:144-183.
 29. Mohamed, N. A., Randles, J. W., and Francki, R. I. B. 1973. Protein composition of tomato spotted wilt virus. *Virology* 56:12-21.
 30. Nossal, G. J. V., Pike, B. L., Teale, J. M., Layton, J. R., Kay, T. W., and Batty, F. L. 1979. Cell fractionation methods and the target cells for clonal abortion of B lymphocytes. *Immunol. Rev.* 43:185-216.
 31. Paliwal, Y. C. 1976. Some characteristics of the thrip vector relationship of tomato spotted wilt virus in Canada. *Can. J. Bot.* 54:402-405.
 32. Reddy, D. V. R., and Wightman, J. A. 1988. Tomato spotted wilt virus: thrips transmission and control. *Adv. Dis. Vector Res.* 5:203-220.
 33. Sherwood, J. L., Sanborn, M. R., Keyser, G. C., and Myers, L. D. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. *Phytopathology* 79:61-64.
 34. Smith, R. T., and Bridges, R. A. 1958. Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens. *J. Exp. Med.* 108:227-250.
 35. Storey, H. H. 1931. The bearing of insect-vectors on the differentiation and classification of plant viruses. Pages 471-479 in: *Cong. Int. Pathol. Comp.*, 2nd.
 36. Szewczuk, M. R., and Siskind, G. W. 1977. Ontogeny of B-lymphocyte function. III. In vivo and in vitro studies on the ease of tolerance induction in B lymphocytes from fetal, neonatal and adult mice. *J. Exp. Med.* 145:1590-1601.