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# The Antibody Response of Mice to Murine Leukemia Virus in Spontaneous Infection: Absence of Classical Immunologic Tolerance

**(AKR mice/complement-fixing antibodies/lymphocytic choriomeningitis virus/ immunofluorescence/glomerular deposits of antigen–antibody complexes)**

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**ABSTRACT** **Gross murine leukemia virus is the etiologic agent of spontaneous leukemias of AKR mice. Despite the persistence of the Gross virus throughout their life, these mice are not immunologically tolerant to the virus. Specific antibodies to Gross antigens can be detected in the kidney where they have been deposited in the glomeruli, apparently in the form of Gross antigen–antibody complexes.**

To explain the life-long persistence of certain viruses in infected animals, Burnet and Fenner (1) proposed the hypothesis of immunologic tolerance. They suggested that after a generalized nonfatal infection of the embryo, the animal, after birth, would be incapable of producing antibody to the same infecting agent. Observations with lymphocytic choriomeningitis (LCM; refs. 2–6) and tumor viruses (7–10) provided the main support for this concept. In both of these infections, virus is transmitted vertically and a permanent viral carrier state ensues. Characteristically, virus is detectable in blood and organs throughout life, while free circulating antibody to viral antigen(s) is not.

Previously we reported that mice infected with LCM virus either *in utero* or in the newborn period were not "immunologically tolerant", as they were capable of making an immune response to the virus (11–13). Now we report that neither immunologic nor clinical tolerance to Gross murine leukemia virus-(GMuLV-) related antigens occurs in AKR mice. Such mice are naturally infected *in utero* with GMuLV and carry large amounts of virus throughout their life (14–16).

## MATERIALS AND METHODS

AKR mice of both sexes were obtained from Jackson Laboratories, Bar Harbor, Me. At 3, 6, and 9 months, blood was obtained by orbital sinus puncture for serologic and hematologic studies. Testing of AKR mice revealed that over 90% of the mice were positive for Gross soluble antigen at 3 months; all mice were positive for the Gross antigen by 6 months. The assay for the Gross soluble antigen, by use of sera from C57BL/6 mice inoculated with $K_{36}$ cells, has been described (17, 18). Other AKR mice were killed at similar times and their tissues were examined for the presence of viral antigen, host immunoglobulin (Ig), third complement component (C3), albumin, and fibrinogen by immunofluorescent techniques as outlined (12). Details as to preparation of monospecific antisera and labeling with fluorescein isothiocyanate have been published (12). Rat antiserum containing antibody to Gross viral envelope antigens was kindly supplied by Dr. Wallace Rowe of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. It was absorbed with purified mouse Ig.

Kidneys from 25–30 mice showing heavy IgG deposits in their glomeruli were homogenized and the Ig was eluted as reported (12). Renal eluate and undiluted plasma (at a concentration of 200 $\mu$g of IgG/ml) were assayed for the presence of fluorescent binding and complement-fixing antibodies to Gross antigens. In the fluorescent assay, test material was layered on 4-$\mu$m thick sections of AKR thymus and after 1 hr of incubation, tissue was washed and then stained with rabbit anti-mouse 7S IgG conjugated with fluorescein isothiocyanate. The complement-fixing assay was a modification of the procedure reported by Wasserman and Levine (19). The Gross antigen used was made according to the method of Hartley *et al.* (20). After an 18-hr incubation of renal eluate, antigen, and complement at 4°C, $2 \times 10^7$ sensitized sheep erythrocytes, labeled with $^{51}Cr$ (228Ci/gmCr) were added and the resultant mixture was gently shaken for 1 hr at 37°C. Presence or absence of complement fixation was determined by calculation of the amount of $^{51}Cr$ released in the fluid phase after centrifugation at $700 \times g$ for 10 min.

In addition, we looked for antibody to surface antigen(s) of cells infected with Gross leukemia virus. Briefly, $5 \times 10^6$ washed, viable E♂G2 cells (leukemia cells from C57 BL/6 mice, ref. 21) were mixed with Ig eluted from AKR mouse kidneys (100 $\mu$g of Ig in 0.5 ml of Eagle's balanced salt solution). After washing, the cells were incubated first with F(ab')2 hybrid antibody against mouse IgG–southern bean mosaic virus and then with southern bean mosaic virus. For detailed information on the surface antigens of cells infected with Gross leukemia virus and hybrid antibody procedures see refs. 21–23.

## RESULTS

### Gross virus carrier state

*Immunofluorescent Study.* Kidney tissues from 10 male and 10 female 3-, 6-, and 9-month-old AKR mice were studied for the presence and distribution of host IgG, third component of complement (C3), albumin, and fibrinogen and for Gross

Abbreviations: LCM, lymphocytic choriomeningitis; GMuLV, Gross murine leukemia virus; C3, third component of complement.

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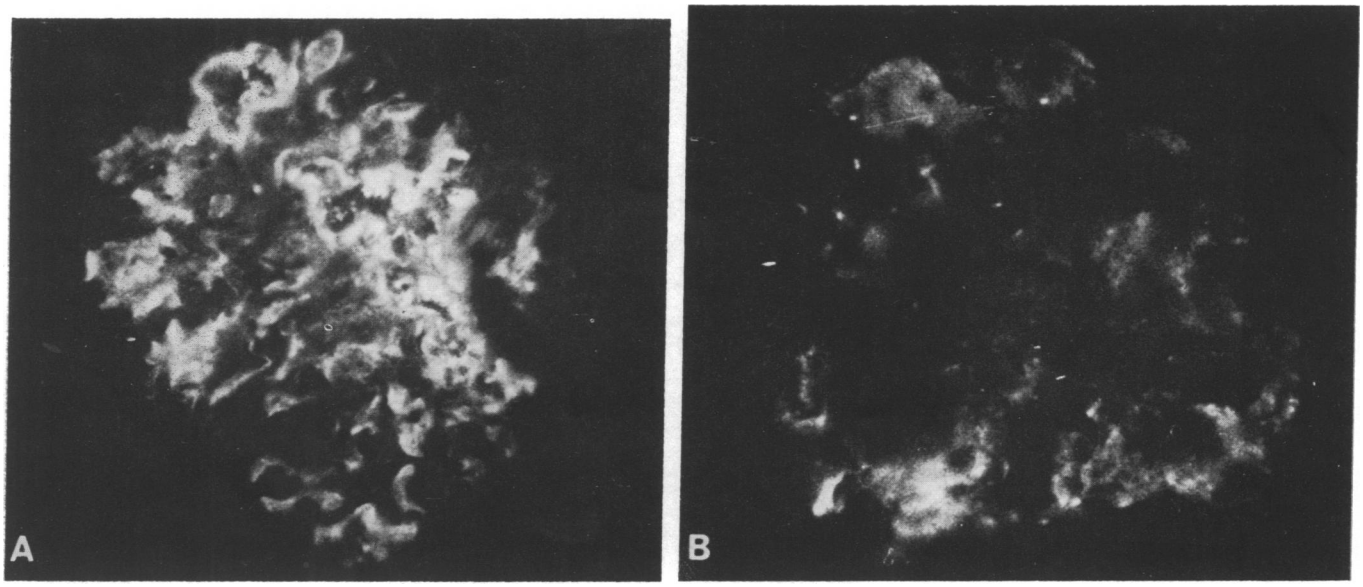


FIG. 1. Renal glomerulus from a 6-month-old AKR mouse. (*A*) Glomerulus stained with fluorescein-conjugated rabbit antibody to mouse 7S IgG. IgG outlines the glomerular basement membrane in peripheral areas and is heavily deposited in the mesangia. (*B*) Glomerulus stained with rat antibody to Gross antigen and then rabbit antibody to rat 7S IgG–fluorescein isothiocyanate. Both rabbit and rat sera were first absorbed with mouse Ig. Gross antigen is deposited in the glomerular basement membrane and mesangia.

antigens by direct and indirect immunofluorescent assays, respectively. In 55% of 3-month, 80% of 6-month, and 95% of 9-month-old mice, host IgG and C3, but not albumin or fibrinogen, were found deposited in a granular pattern in the mesangial areas and peripheral walls of glomerular capillaries (Fig. 1*A*). In contrast to the restriction of IgG and C3 deposits to the glomeruli, Gross antigens were seen in glomeruli (Fig. 1*B*) and also in convoluted tubules, connective tissue, and arterial endothelium. Glomerular-bound antigens were most easily seen after elution of Ig from the tissue section by incubation with 0.12 M glycine (pH 3.0) for 45 min at 37°C before immunofluorescent staining.

Gross antigen(s) detected with rat antiserum was found in most tissues studied, being most concentrated in the thymus, lymph nodes, spleen, and kidney. In comparison, both IgG and C3 accumulated only in renal tissue. Occasionally, all

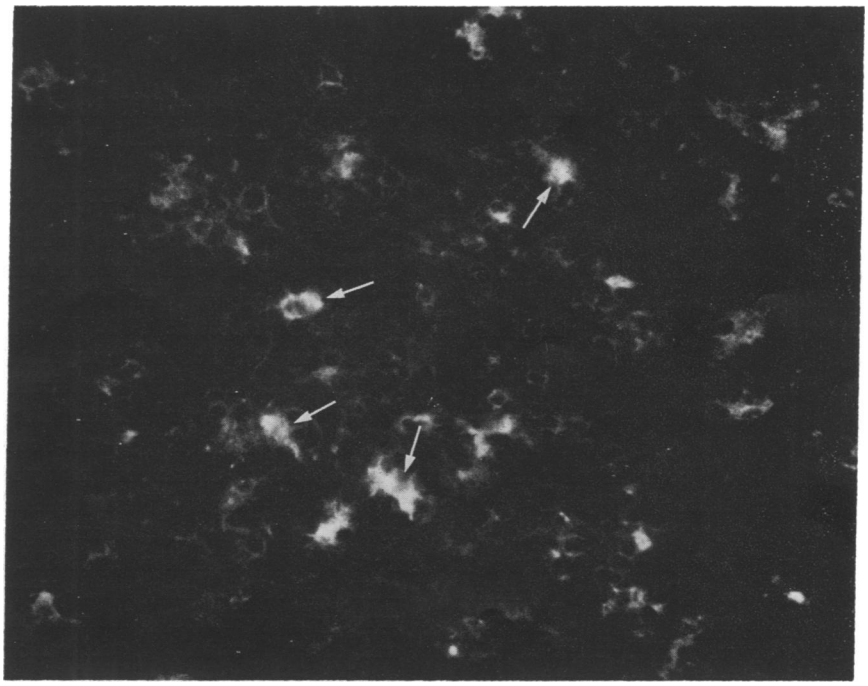

FIG. 2. Indirect immunofluorescent assay for the detection of antibody to Gross antigen(s). Kidney eluates from AKR mice were layered on top of AKR thymic tissue sections. After washing, rabbit antibody to mouse 7S IgG–fluorescein isothiocyanate was added. *Arrows* point to several thymic cells containing cytoplasmic granules stained a brilliant apple green. Similar treatment of SWR/J thymic tissue failed to reveal positive staining.

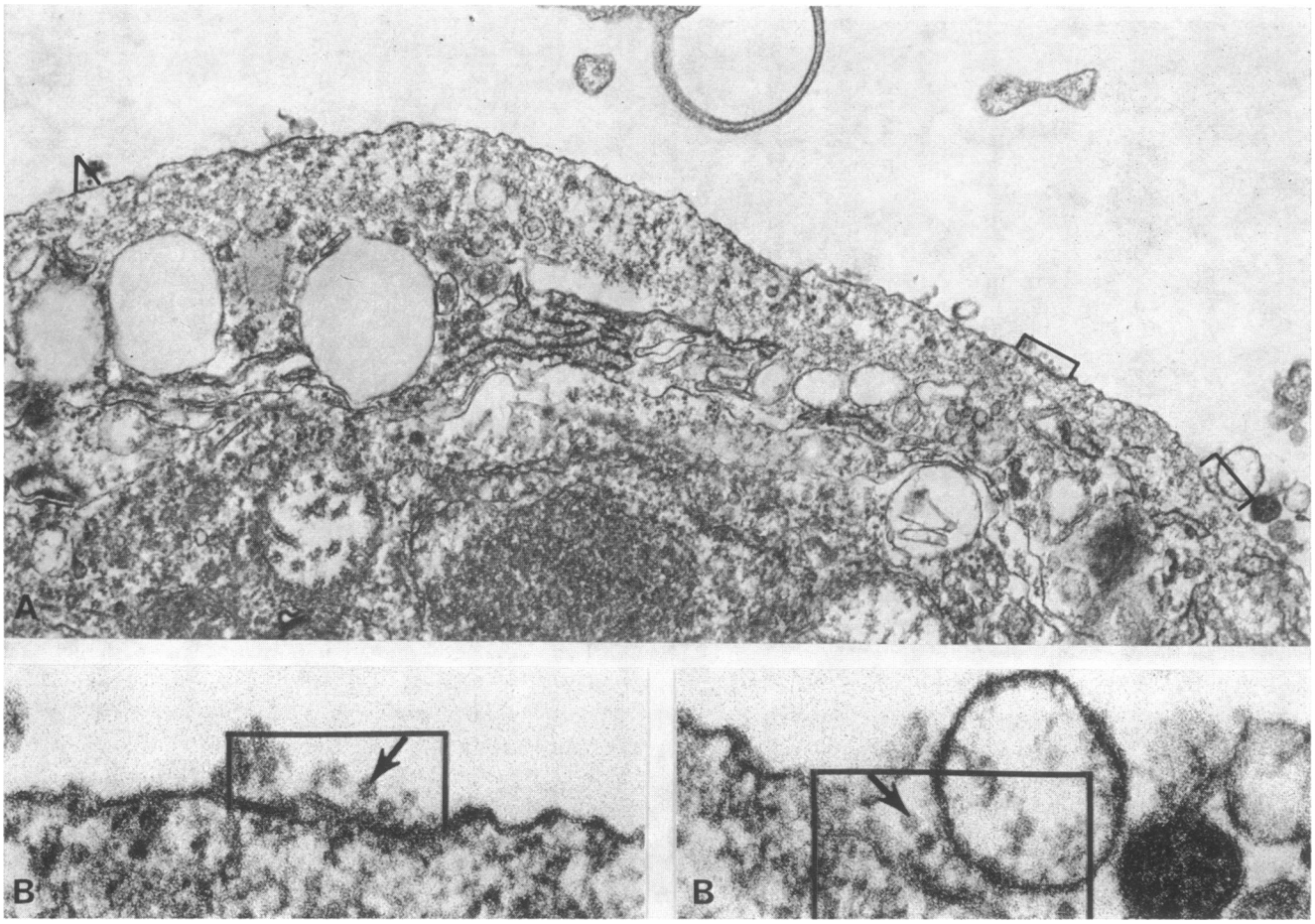


FIG. 3. Gross cell-surface antigen(s) on C57BL/6 leukemia cells induced by passage-A Gross virus. Cells were initially incubated with the eluate from AKR kidneys and then with hybrid antibody by use of southern bean mosaic virus as a marker (see *Methods*). (*A*) Low-power electron micrograph showing three small areas marked by southern bean mosaic virus. ×6,000. (*B*) Enlargement of areas. *Arrows* point to southern bean mosaic virus ×252,000.

four plasma proteins were deposited in necrotic foci of the liver, heart, and brain.

*Detection of Antibody to GMuLV Antigens.* Antibodies to GMuLV antigens were found in the Ig eluted from AKR kidneys. By use of indirect immunofluorescence, positive staining of thymus cells from AKR mice occurred after the addition of AKR kidney eluate (Fig. 2), but not with AKR plasma or similarly prepared eluates from LCM or lactic dehydrogenase virus-infected mice. At similar concentrations, the eluate from AKR mice failed to stain thymus tissue from control SWR/J mice. In addition, the Ig eluted from AKR glomeruli specifically fixed complement in the presence of Gross antigen (Table 1). Only 5% of the total $^{51}Cr$ from sensitized sheep erythrocytes was released after incubation with Gross antigen, eluted Ig, and complement, as compared to 57% when eluted Ig, 68% when Gross antigen, or 77% when complement alone were added. Furthermore, the presence of antibody to surface antigen of cells infected with Gross virus was demonstrated by immunoelectron microscopy by use of the hybrid antibody assay. Fig. 3 shows the presence of antibody to antigens on the surface of E♂G2 leukemia cells that are infected with Gross virus after the incubation of these cells with the eluate from AKR kidneys. The AKR kidney eluate did not react with Gross viral envelope antigens by this method. Antibody to surface antigen of cells infected with Gross antigen was not detected in the plasma of AKR mice.

## DISCUSSION

GMuLV has been implicated by several investigators as the etiologic agent in spontaneous leukemias of AKR mice (14–16). Despite life-long persistence of virus, these mice make antibody responses to GMuLV antigens. Presumably, because of an excess of viral antigen(s), free antibody to these antigens is not detected in the circulation. However, finding host Ig, C3, and Gross antigen in the glomeruli suggests that circulating Gross antigen–anti-Gross antibody complexes probably occur and are subsequently deposited in the glomerular capillary walls and mesangia. Such antibodies can be recovered and then assayed directly. Our data indicate that at least two distinct antibodies to GMuLV are being made by AKR mice. First, complement-fixing antibodies appear immunologically related to internal viral components (24, 25) and, second, antibody to viable E♂G2 cells is directed towards Gross leukemia cell-surface antigens (26). Others (27) have shown that sera from AKR mice contain cytotoxic antibodies both for cells from a leukemia induced by passage A virus, and spontaneous AKR leukemia cells after immunization with isogenic $K_{36}$ cells, suggesting the presence of anti-

bodies to Gross antigens. However, the possibility that these antibodies might also be directed to histoincompatibility or autoantigenic determinants was not excluded.

Previous failures to detect antibody to Gross virus in AKR mice suggested that these mice were immunologically tolerant to the virus (7, 9). However, it is now clear that *in utero* infection with Gross virus does not induce complete tolerance to Gross antigens determined by the Gross viral genome, even in the presence of persistent viral infection. In this regard, it is doubtful whether "classic" immunologic tolerance occurs in any viral infection. In those animal models, both natural and experimental, previously thought to represent examples of immunologic tolerance, it is now apparent that specific antibody to virus occurs, i.e., LCM virus (11, 12) and lactic dehydrogenase virus (28–31), Moloney sarcoma virus (32) infection of mice, Aleutian disease of mink (33, 34), and equine infectious anemia (35). Furthermore, the observations made on human infants infected with rubella or cytomegalic virus in fetal life indicate that these babies too are not immunologically tolerant in spite of persistent infection (36–39).

In addition, a cellular response to viruses causing chronic infection is made. While certain chronic viral infections *per se* may suppress the host's immune response (both cellular and humoral) to a wide range of nonviral antigens (reviewed in ref. 40), the activation of lymphoid cells from chronically infected animals by the infecting virus can occur. Wahren and Metcalf (41) reported a cytotoxic reaction after the addition of preleukemic AKR lymphoid cells to AKR tissues, indicating that some lymphoid cells acquired immunological reactivity to virus-induced antigens. Similarly, cells sensitized against LCM viral antigen(s) can be found in mice persistently infected with LCM virus (42–44). However, these sensitized lymphoid cells had less than half the cytotoxic and colony-inhibiting activity of similar numbers of lymphoid cells harvested from immune mice (44). Why these viruses are able to persist is not known; whether or not early infection induces an immunological hyporesponsiveness to the infecting agent is uncertain, since quantitative measurements in the presence of excess viral antigen are not at present possible. If a lessening of humoral or cellular immunity occurs, it might make the host less able to reject virus or virus-infected cells. However, it should be remembered that presumably very small immune responses, such as those made by individuals with various forms of immunodeficiency, appear to provide adequate protection against most of the common acute viral infections. It is also possible that the basis for persistent viral infection might be more the result of unique behavior of the virus rather than immunologic hyporesponsiveness of the host. These agents, for unknown reasons, may be able to elude usual immune responses ordinarily effective against agents causing acute infections.

Finally, virus-induced immunopathology was an important factor in the pathogenesis of glomerulonephritis in AKR mice. Glomerular deposits of Gross antigen–Gross antibody complexes were found in some mice by 3 months of age and increased with time.† By 9 months of age, 26% of AKR mice had proteinuria, 70% histologic and 95% immunofluorescent evidence of nephritis, and 10% apparently died from this immune complex disease, indicating that clinical tolerance to Gross antigens was nonexistent.

TABLE 1. *Antibody to Gross antigen in AKR mice**

| | Counts of $^{51}Cr$/30 sec | | |
|---|---|---|---|
| | Supernatant | Cell sediment | %† |
| Gross antigen‡ + 4 units murine leukemia antisera§ + C | 685 | 12391 | 5.2 |
| + AKR eluate¶ + C | 638 | 12768 | 4.7 |
| + C | 9011 | 4052 | 68.9 |
| + LCM carrier eluate‖ + C | 8781 | 4427 | 66.4 |
| AKR eluate + C | 7890 | 5859 | 57.3 |
| C only | 9961 | 2939 | 77.2 |

* Micro complement (C) fixation assay (19), in which 1.2 median complement hemolytic units ($C'H_{50}$) were used.

† % of $^{51}Cr$ released in fluid phase (supernatant) over total $^{51}Cr$ incorporated into sensitized erythrocytes.

‡ Complement-fixing Gross antigen prepared by the method of Hartley *et al.* (20).

§ Antiserum against Moloney sarcoma virus was prepared in tumor-bearing Fisher rats kindly supplied by Dr. R. Wilsnack of Huntington Research Center.

¶ Eluate from AKR mouse kidneys at a concentration of 200 μg of IgG/ml.

‖ Eluate from SWR/J mouse kidneys at a concentration of 200 μg of IgG/ml. These mice were persistently infected with LCM virus and showed heavy IgG glomerular deposits.

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1. Burnet, F. & Fenner, F. (1949) in *The Production of Antibodies* (Macmillan and Co. Ltd, Melbourne), 2nd Ed, p. 104.
2. Traub, E. (1935) *Science* **81**, 298.
3. Traub, E. (1936) *J. Exp. Med.* **63**, 847.
4. Traub, E. (1936) *J. Exp. Med.* **64**, 183.
5. Traub, E. (1938) *J. Exp. Med.* **68**, 229.
6. Traub, E. (1939) *J. Exp. Med.* **69**, 801.
7. Gross, L. (1961) in *Oncogenic Viruses* (Pergamon Press, N.Y.), pp. 153–231.
8. Ruben, H. (1962) *Nature* **195**, 342.
9. Axelrad, A. (1963) *Nature* **199**, 80.
10. Klein, E., & Klein, G. (1966) *Nature* **209**, 163.
11. Oldstone, M. B. A. & Dixon, F. J. (1967) *Science* **158**, 1193.
12. Oldstone, M. B. A. & Dixon, F. J. (1969) *J. Exp. Med.* **129**, 483.
13. Oldstone, M. B. A. & Dixon, F. J. (1970) *J. Immunol.* **105**, 829.
14. Old, L. J., Boyse, E. A. & Stockert, E. (1965) *Cancer Res.* **25**, 813.
15. Wahren, B. (1965) *Nature* **205**, 409.
16. Geering, G., Old, L. & Boyse, E. (1966) *J. Exp. Med.* **124**, 753.
17. Aoki, T., Old, L. J. & Boyse, E. A. (1966) *Nat. Cancer Inst. Monogr.* **22**, 449.
18. Aoki, T., Boyse, E. & Old, L. (1968) *J. Nat. Cancer Inst.* **41**, 89.
19. Wasserman, E. & Levine, L. (1961) *J. Immunol.* **87**, 290.
20. Hartley, J., Rowe, W., Capp, W. & Huebner, R. (1965) *Proc. Nat. Acad. Sci. USA* **53**, 931–938.
21. Aoki, T., Boyse, E. & Old, L. (1966) *Cancer Res.* **26**, 1415.
22. Hämmerling, U., Aoki, T., deHarven, E., Boyse, E. A. & Old, L. J. (1968) *J. Exp. Med.* **128**, 1461.

† Oldstone, M. B. A., Tonietti, G. & Dixon, F. J., manuscript in preparation.

23. Hämmerling, U., Aoki, T., Wood, H. A., Old, L. J., Boyse, E. A. & deHarven, E. (1969) *Nature* **223,** 1158.
24. Gregoriades, A. & Old, L. (1969) *Virology* **37,** 189.
25. Huebner, R. J. (1967) *Proc. Nat. Acad. Sci. USA* **58,** 835–842.
26. Aoki, T., Boyse, E. A., Old, L. J., deHarven, E., Hämmerling, U. & Wood, H. A. (1970) *Proc. Nat. Acad. Sci. USA* **65,** 569–576.
27. Doré, J. F., Ajuria, E. & Mathe, G. (1970) *Rev. Eur. Études Clin. Biol.* **XV,** 81.
28. Notkins, A. L., Mahar, S., Scheele, C. & Goffman, J. (1966) *J. Exp. Med.* **124,** 81.
29. Notkins, A. L., Mage, M., Ashe, W. K. & Mahar, S. (1968) *J. Immunol.* **100,** 314.
30. Oldstone, M. B. A. & Dixon, F. J. (1971) *J. Immunol.* **106,** 1260–1263.
31. Porter, D. & Porter, H. (1971) *J. Immunol.* **106,** 1264.
32. Hirsch, M., Allison, A. & Harvey, J. (1969) *Nature* **223,** 739.
33. Porter, D. & Larsen, A. (1967) *Proc. Soc. Exp. Biol. Med.* **126,** 680.
34. Porter, D., Larsen, A. & Porter, H. (1969) *J. Exp. Med.* **130,** 575.
35. Banks, K. & Henson, J. (1969) *Fed. Proc.* **28,** 752.
36. Alford, C. A., Jr., Neva, F. A. & Weller, T. H. (1964) *N. Engl. J. Med.* **271,** 1275.
37. Phillips, C. A., Melnick, J. L., Yow, M. D., Bayatpour, M. & Burkhardt, M. (1965) *J. Amer. Med. Ass.* **193,** 1027.
38. Plotkin, S. A., Dudgeon, J. A., & Ramsay, A. M. (1963) *Brit. Med. J.* **2,** 1296.
39. Weller, T. H., Alford, C. A., Jr. & Neva, F. A. (1964) *N. Engl. J. Med.* **270,** 1039.
40. Notkins, A. L., Mergenhagen, S. E. & Howard, R. J. (1970) *Annu. Rev. Microbiol.* **24,** 525–538.
41. Wahren, B. & Metcalf, D. (1970) *Clin. Exp. Immunol.* **7,** 373.
42. Lundstedt, C. (1969) *Acta Path. Microbiol. Scand.* **75,** 139.
43. Oldstone, M. B. A., Habel, K. & Dixon, F. J. (1969) *Fed. Proc.* **28,** 429.
44. Oldstone, M. B. A. & Dixon, F. J. in *Sixth Int. Symp. of Immunopathol.*, in press.