CELLULAR IMMUNITY IN VACCINIA INFECTION OF MICE

Anti-thymocyte Serum Effects on Primary and Secondary Responsiveness

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Host response to viral infection may take many forms including stimulation of humoral and cellular immune mechanisms and production of interferon. The importance of each of these mechanisms in individual viral infections has been the subject of several recent reviews (1–4). The role of cellular immunity in vaccinia infection was first suggested by von Pirquet in 1907 (5), and has been studied extensively in recent years (2, 6–9). Its role relative to those of antibody and interferon, however, remains unresolved (6–11). Many of the investigations concerning host response in vaccinia infection have employed immunosuppressants in an attempt to isolate one or more reactive mechanisms for particular attention (6, 8). Among the most potent known suppressants of cellular immunity are anti-lymphoid sera (12). Such sera have been used to study the relative role of cellular responses in several viral infections, including lymphocytic choriomeningitis, yellow fever, Rauscher leukemia, Moloney leukemia, polyoma, herpes simplex, rabies, and influenza (13–20).

In the present report, rabbit anti-mouse thymocyte (RAMT) serum was demonstrated to increase morbidity and mortality induced by vaccinia infection in mice after intravenous virus administration. However, it was found to have no effects on the course of primary or secondary infection after intracerebral inoculation. The suppressive effects of RAMT serum on host resistance to intravenously administered virus did not appear to be related to reduced interferon or antibody production, but rather to markedly diminished cellular immune responsiveness.

Materials and Methods

Mice.—Male ICR mice, 3–4 wk old, were used in all experiments.

Viruses.—Vaccinia virus (IHD-E neurotropic strain) was obtained from Dr. W. A. Cassel, Emory University. A 20% mouse brain suspension contained $10^{7.0}$ LD_{50} per 0.03 ml. Vaccinia

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virus titrations of the original inoculum and of infected organs were performed by intracerebral inoculation of 3-wk old ICR mice. Mengo virus in infected L cell supernatant fluid was obtained from Dr. W. Ashe, Emory University. It contained $10^{7.0}$ plaque-forming units (PFU) per ml.

Anti-Thymocyte Sera.—RAMT serum was prepared as described by Levey and Medawar (12) by immunizing rabbits with two intravenous inoculations of 10^9 dispersed viable ICR mouse thymus cells. Individual sera were pooled and absorbed against erythrocytes. The effectiveness of the pool was assayed by its ability (*a*) to diminish peripheral blood lymphocyte counts by 50% within 4 hr after a single intraperitoneal inoculation of 0.3 ml and (*b*) to prevent clinical and histologic signs of LCM virus infection in ICR mice given three intraperitoneal inoculations (0.05 ml) of RAMT serum at 3-day intervals before virus challenge. This latter test has been found in our laboratory to be a reliable assay of suppression of cellular immunity (14). All RAMT and normal rabbit sera were shown to be free of anti-vaccinia activity by intracerebral virus neutralization assays in mice.

TABLE I

Effects of Rabbit Anti-mouse Thymocyte (RAMT) Serum and Normal Rabbit Serum (NRS) on Primary Vaccinia Infection in Mice

Virus dilution	Route of inoculation	RAMT		NRS	
		No. of mice	Mortality	No. of mice	Mortality
			%		%
10^{-1}	i.v.	20	80	20	5
10^{-2}	i.v.	10	50	10	0
10^{-3}	i.v.	10	20	10	0
10^{-6}	i.c.	12	100	12	100
10^{-7}	i.c.	12	33	9	45
10^{-8}	i.c.	13	15	10	0

All animals received 0.3 ml of RAMT serum or NRS intraperitoneally on days −6, −3, 0, +3, +6, and vaccinia virus on day 0. Intracerebral (i.c.) virus doses were 0.03 ml; intravenous (i.v.) doses were 0.05 ml.

Experimental Design

Primary Response.—148 mice were divided into two groups (Table I). One group was inoculated with 0.3 ml of RAMT serum every 3rd day beginning 6 days before virus inoculation, and the other received normal rabbit serum (NRS) according to the same schedule. On day zero, mice were inoculated either intravenously or intracerebrally with vaccinia virus. Intravenous doses were 0.05 ml and dilutions in Hanks' medium were 10^{-1}–10^{-8}; intracerebral doses were 0.03 ml and dilutions were 10^{-6}–10^{-8}. Animals were observed daily for signs of vaccinia infection, i.e., lethargy, dehydration, ataxia, ruffled fur; in addition intravenously inoculated mice were observed for development of tail lesions as described by Boyle et al. (21). Gross and light microscopic examinations and virus titrations were performed on organs from representative animals of each group. Organs were fixed in buffered formalin, and 4-μ sections were stained with hematoxylin and eosin.

Interferon analyses were performed on serum from 22 animals intravenously inoculated with vaccinia virus (10^{-1}), according to a modification of the plaque reduction technique of Glasgow (10, 22). Serum samples collected 12 hr after virus inoculation were acidified (pH 2) for 48

hr, brought back to neutrality, and incubated overnight on L cell monolayers in prescription bottles. 100 PFU of Mengo virus per bottle were adsorbed to the cultures for 30 min, and nutrient agar overlay containing 200 μg/ml of protamine sulfate was added. After 48 hr of incubation, 1.0 ml neutral red (1:1000) was added and plaques were counted. Per cent inhibition was plotted on arithmetic probability paper, and the reciprocal of the dilution giving 50% inhibition was used to represent the units of interferon per unit volume of serum.

Survivors of intravenous virus inoculation were tested by a microtechnique for vaccinia hemagglutination inhibition (HI) antibodies 3 wk after initial viral challenge. These animals were subsequently rechallenged intracerebrally with 10^3 LD_{50} of vaccinia virus.

Secondary Response.—136 mice were vaccinated twice by tail scarification with 10^6 LD_{50} of vaccinia virus. 1 month after the second vaccination these mice were divided into two groups one which received RAMT serum (72 animals), the other NRS, according to the same dosage schedules as described under *Primary response.* On day zero the animals were challenged with 10^3 to 10^6 LD_{50} intracerebrally (10–25 animals per dilution). Mice were observed daily for signs of infection, and gross and light microscopic examinations were performed on organs from dying animals. Representative animals from both groups were tested for vaccinia HI antibodies 1 day before and 3 days after virus rechallenge (24 animals tested).

RESULTS

A. Primary Response

Primary Response to Intravenous Administration.—RAMT serum markedly altered the course of intravenous vaccinia infection, resulting in an increased morbidity and mortality (Fig. 1). At a 10^{-1} virus dilution, 20% of the RAMT serum–treated animals survived compared to 95% of the NRS group; at 10^{-2}, 50% of the RAMT serum group and 100% of the NRS group survived. Numbers and size of tail lesions were also increased in RAMT serum–treated animals. The number of pocks was approximately four times greater in RAMT serum–treated animals than in the NRS group (Table II), and the pock size was enlarged by approximately 50%.

Histologic and Virologic Observations.—Tail lesions were composed histologically of acute necrotizing inflammatory areas with accumulated cellular debris consisting of degenerating polymorphonuclear leukocytes and plasma cells (Fig. 3). In RAMT serum–treated animals these areas were larger and less well circumscribed; frequently eosinophilic cytoplasmic inclusions and interstitial edema were present. Overlying epidermis, though generally intact, was frequently hypertrophied in areas of dermal involvement. RAMT serum treatment also was associated with widespread virus multiplication and destruction in other organs. Both groups developed areas of interstitial necrotizing pneumonitis (Fig. 4) with lung virus titers of 10^4–10^5 LD_{50} per 0.03 ml, whereas only RAMT serum–treated mice had detectable virus and demonstrable lesions in other organs. Titers in the RAMT serum group ranged from $10^{2.3}$ to $10^{4.0}$ LD_{50} per 0.03 ml in liver, spleen, and kidney, and necrotic foci were found in liver, meninges (Fig. 5), and brain parenchyma. Occasionally a necrotizing exudative mitral valvulitis was found in RAMT serum–treated animals (Fig. 6). Spleen and lymph nodes of RAMT serum–treated mice exhibited depletion of small

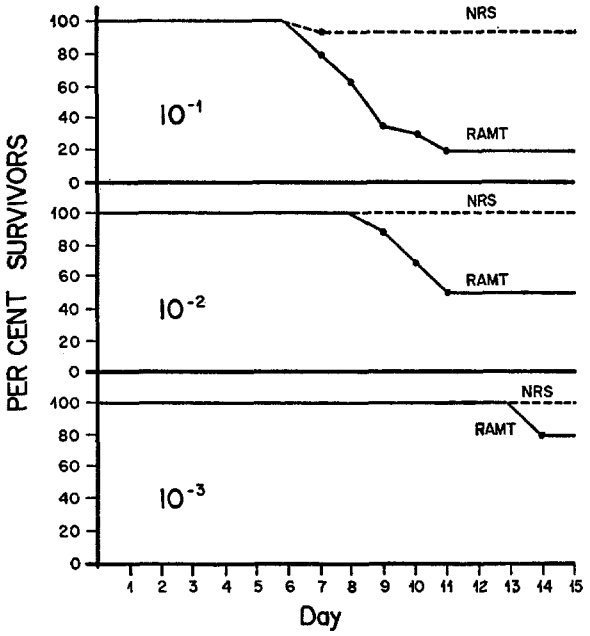

FIG. 1. Survival curves demonstrating effects of RAMT serum and NRS on mortality after intravenous administration of vaccinia virus in doses of 10^{-1}–10^{-3}.

TABLE II

Effect of RAMT Serum and NRS on Tail Pocks Induced by Intravenous Vaccinia Virus

Pretreatment*	No. of animals	Total pocks	Mean No. pocks
RAMT, 10^{-2}	7	123	17.6
NRS, 10^{-2}	5	21	4.2
RAMT, 10^{-3}	10	27	2.7
NRS, 10^{-3}	8	5	0.6

* All animals received 0.3 ml of either RAMT serum or NRS intraperitoneally on days −6, −3, 0, +3, +6; virus dilutions 10^{-2} and 10^{-3} were given intravenously in doses of 0.05 ml on day 0.

lymphocytes, a change characteristically seen in RAMT serum–treated animals (13).

Interferon Measurements.—No differences were found among animals that received RAMT serum, NRS, or no treatment (22 animals tested). The mean titers in all three groups were between 20–25 units/ml.

Serologic Observations.—No differences were found in levels of vaccinia HI

antibody between the groups pretreated with either RAMT serum or NRS (16 animals tested). Median titers in both groups were 1:64 with ranges from 1:16 to 1:128. All animals were resistant to rechallenge with 10^3 LD_{50} of vaccinia virus administered intracerebrally.

Primary Response to Intracerebral Administration.—Whereas RAMT serum had marked effects after intravenous virus administration, it had no effect on the course of infection after intracerebral inoculation (Fig. 2). Both groups had

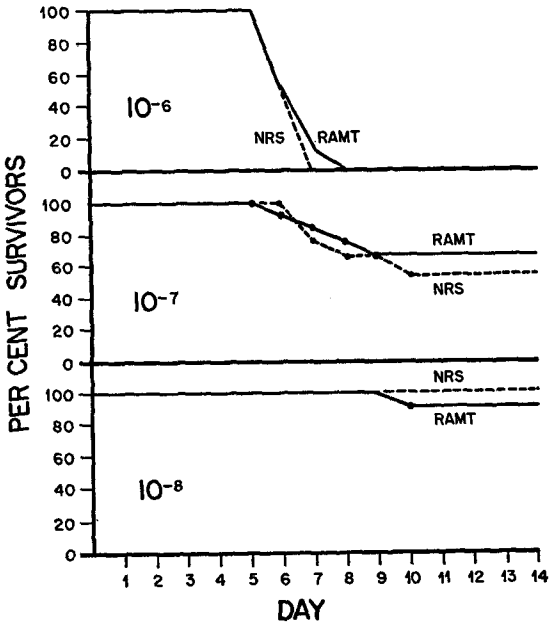

FIG. 2. Survival curves demonstrating effects of RAMT serum and NRS on mortality after intracerebral administration of vaccinia virus in doses of 10^{-6}–10^{-8}.

severe necrotizing meningitis and encephalitis similar to that after primary intravenous inoculation (Fig. 5) with gliosis, neuronal degeneration, foamy cells, acute inflammatory cells, and occasional hemorrhages. Except for depletion of small lymphocytes in nodes and spleens of RAMT serum–treated animals, all other organs were normal in appearance in both groups.

B. Secondary Response

RAMT serum did not alter the course of infection in animals immunized with vaccinia virus by tail scarification and challenged intracerebrally. Mortality in both RAMT serum and NRS groups was equivalent at all virus concentrations

tested, even when 10^6 LD_{50} of vaccinia virus was administered intracerebrally. The total mortality was 32% in the RAMT serum group (23/72) and 23% in the NRS group (15/64); these differences are not significant at the 0.05 level by the chi-square test. On light microscopic histological examination severe necrotizing meningitis and encephalitis were demonstrated in dying animals. No significant differences were found in prechallenge HI antibody level or in post-challenge antibody response between RAMT- and NRS-treated groups or between dying animals and survivors (24 animals tested). The average titers before challenge in all groups were from 1:32 to 1:64 and after challenge from 1:64 to 1:128.

DISCUSSION

The course of vaccinia infection in vivo depends on various factors including strain of virus (23), route of inoculation (24, 25), species of animal (2), and status of host defense mechanisms (2, 6–11). Among host factors shown to participate in reactivity to this virus are circulating antibody (11, 26), interferon (6, 10), and cell-mediated immune responses (2, 4). No unanimity of opinion has developed regarding the relative roles of the latter two mechanisms in responsiveness to vaccinia virus.

Friedman et al. (6) found that guinea pigs in which skin hypersensitivity and antibody production were blocked by X-irradiation and methotrexate but in which the capacity to produce interferon was preserved recovered from vaccinia infection as rapidly as normal animals. These workers hypothesized that locally produced interferon was instrumental in recovery from infection. However, the guinea pig may not have been a good model because of its inherent resistance to vaccinia virus (2); in addition, absence of skin reactivity may not necessarily have reflected absence of cellular immunity since skin tests may be relatively insensitive assays of this response (7, 27). In contrast to the work of Friedman et al., Pincus and Flick reported a series of experiments that demonstrated a role for cellular immunity in primary responsiveness to vaccinia virus (7–9). They found that (*a*) the ability of various inbred strains of rabbits to develop rapid and severe reactions to vaccinia was directly proportional to their ability to mount delayed hypersensitivity to other antigens, and (*b*) techniques that inhibited the expression of cellular immunity, e.g. inoculation of "anti-mononuclear" cell serum locally or the induction of neonatal immunologic unresponsiveness, inhibited the primary vaccinia lesion. The neonatally unresponsive rabbits frequently developed disseminated vaccinia after intradermal infection; autopsy findings in these animals were similar to those described above in RAMT serum–treated mice inoculated intravenously with vaccinia virus.

In humans progressive vaccinia has been closely related to conditions associated with defects of cellular immunity, such as thymic dysgenesis or other less well-categorized deficiency syndromes (3, 11, 28, 29). Local inoculations of

immune leukocytes have resulted in marked regression of such vaccinial lesions (11, 28). Although some workers have proposed that the observed effects may have been a result of interferon production by the transferred leukocytes (10), the balance of evidence suggests that the effects are mediated by cellular immunity. One patient so treated had her own leukocytes assayed for interferon production ability, and the response was adequate (28). Similarly, intact capacities of leukocytes to produce interferon were found in a patient with vaccinia gangrenosa and deficient cellular immunity (29), and in another with thymic dysgenesis (3).

Suppression of cellular immunity is the primary action of anti-thymocyte serum, although the mechanisms by which this is accomplished are not well understood (12). The present study indicates that RAMT serum did not affect the production of interferon in response to vaccinia virus infection. As in other viral infections, it also did not appear to suppress hemagglutination inhibition and complement-fixing antibody formation (13, 14). Hence, its potentiating effect on primary intravenous virus infection must be ascribed to its cellular depressant capacity. This suppression, whether on antigen-processing cells or on sensitized effector lymphocytes, allowed the virus to multiply not only in the lung, its apparent intravenous target organ, but also in the brain, liver, spleen, and skin. Augmented virus multiplication, in turn, resulted in increased tail lesions and organ damage, as well as greater mortality.

Intracerebral inoculation of vaccinia virus resulted in multiplication in the brain and in the development of severe necrotizing meningitis and encephalitis. Spread from the central nervous system to other organs did not appear to be important in the pathogensis after administration by this route, and the course did not appear to be influenced by suppression of cell-mediated immunity. Similar differences between RAMT serum effects on the course of infection after peripheral and intracerebral virus inoculations have been found in the case of herpes simplex virus (17).

Secondary responsiveness to intracerebral challenge was also not affected by RAMT serum pretreatment. Two vaccinations by tail scarification with vaccinia virus provided protection of approximately 75% of the animals against subsequent intracerebral rechallenge, whether the animals had received RAMT serum or not. These results indicate that cellular responsiveness does not play a major role in secondary immunity after intracerebral challenge, although it may be a significant factor in response to challenge by other routes, such as the skin. It is probable that the presence of circulating antibody and possibly of interferon protected the majority of mice against fatal meningitis and encephalitis, since both antibody and interferon have ready access to infected brain (30, 31).

In view of the increasing use of anti-lymphoid sera in human tissue transplantation (32), knowledge concerning the effects of these sera on previously established viral immunity has become important practically (33). Our results

suggest that although susceptibility to primary virus infection may be markedly increased by anti-lymphoid serum treatment, secondary immunity may not be so readily affected. Volkert and Lundstedt, however, have recently demonstrated that preestablished immunity to lymphocytic choriomeningitis virus can be abrogated by treatment with anti-lymphoid sera, despite its inability to reduce complement-fixing antibody formation (15). These findings suggest that cell-mediated responsiveness can play the major part in secondary immunity to some viruses, and be relatively unimportant in immunity to others. The importance of anti-thymocyte serum in reducing established immunity to other common viruses such as varicella and measles remains to be determined.

SUMMARY

Rabbit anti-mouse thymocyte serum suppressed host cell-mediated responsiveness to intravenously administered vaccinia virus, thereby augmenting the morbidity and mortality of this infection. It did not affect either humoral antibody or interferon production in response to vaccinia virus. No effects were noted on primary or secondary immunity to intracerebral virus inoculation.

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BIBLIOGRAPHY

1. Johnson, R. T., and C. A. Mims. 1968. Pathogenesis of viral infections of the nervous system. *New Engl. J. Med.* **278**:23.
2. Allison, A. C. 1967. Cell-mediated immune responses to virus infections and virus-induced tumours. *Brit. Med. Bull.* **23**:60.
3. Gresser, I., and D. J. Lang. 1966. Relationships between viruses and leukocytes. *Progr. Med. Virol.* **8**:62.
4. Mims, C. A. 1964. Aspects of the pathogenesis of virus diseases. *Bacteriol. Rev.* **28**:30.
5. von Pirquet, C. 1907. Klinische Studien uber Vakzination und vakzinale Allergie. Deuticke, Leipzig.
6. Friedman, R. M., S. Baron, C. E. Buckler, and R. I. Steinmuller. 1962. The role of antibody, delayed hypersensitivity, and interferon production in recovery of guinea pigs from primary infection with vaccinia virus. *J. Exptl. Med.* **116**:347.
7. Flick, J. A., and W. B. Pincus. 1963. Inhibition of the lesions of primary vaccinia and of delayed hypersensitivity through immunologic tolerance in rabbits. *J. Exptl. Med.* **117**:633.
8. Pincus, W. B., and J. A. Flick. 1963. Inhibition of the primary vaccinial lesion and of delayed hypersensitivity by an antimononuclear cell serum. 1963. *J. Infect. Diseases.* **113**:15.
9. Pincus, W. B., J. A. Flick, and T. H. Ingalls. 1963. The response of inbred rabbits to vaccinia virus infection. *J. Immunol.* **91**:58.

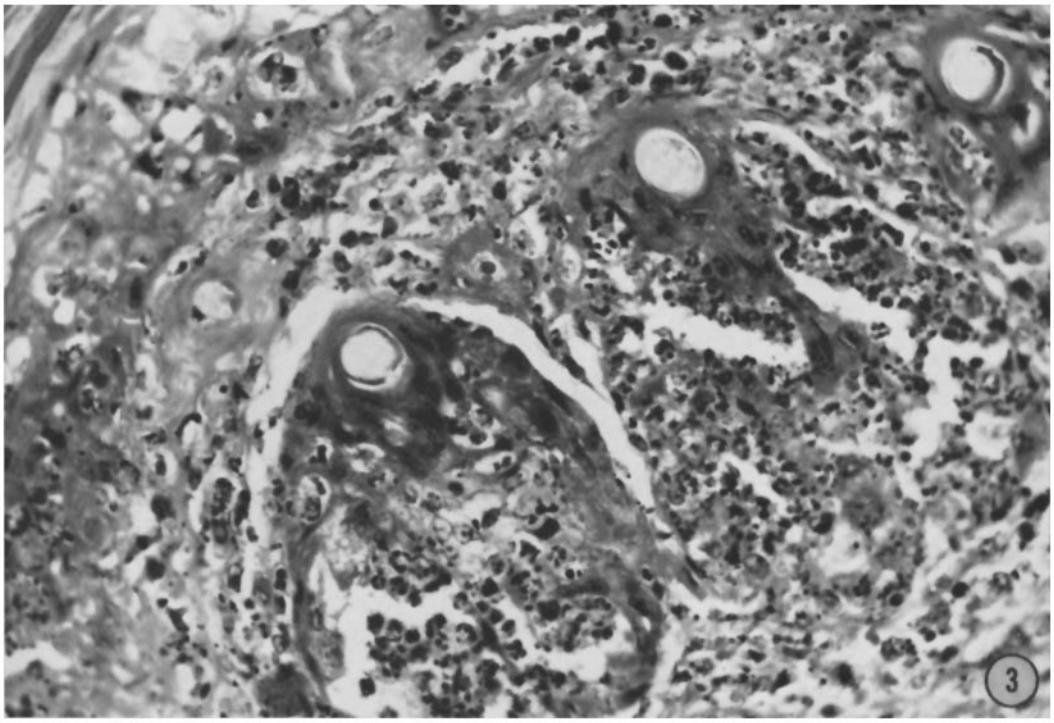
10. Glasgow, L. A. 1966. Leukocytes and interferon in the host response to viral infections: II. Enhanced interferon response of leukocytes from immune animals. *J. Bacteriol.* **91**:2185.
11. Kempe, C. H. 1960. Studies on smallpox and complications of smallpox vaccination. *Pediatrics.* **26**:276.
12. Levey, R. H., and P. B. Medawar. 1966. Nature and mode of action of antilymphocytic antiserum. *Proc. Natl. Acad. Sci. U.S.* **56**:1130.
13. Hirsch, M. S., F. A. Murphy, H. P. Russe, and M. D. Hicklin. 1967. Effects of anti-thymocyte serum on lymphocytic choriomeningitis (LCM) virus infection of mice. *Proc. Soc. Exptl. Biol. Med.* **125**:980.
14. Hirsch, M. S., F. A. Murphy, and M. D. Hicklin. 1968. Immunopathology of lymphocytic choriomeningitis virus infection of newborn mice: anti-thymocyte serum effects on glomerulonephritis and wasting disease. *J. Exptl. Med.* **127**:757.
15. Volkert, M., and C. Lundstedt. 1968. The provocation of latent lymphocytic choriomeningitis virus infections in mice by treatment with antilymphocytic serum. *J. Exptl. Med.* **127**:327.
16. Hirsch, M. S., and F. A. Murphy. 1967. Effects of anti-thymocyte serum on 17-D yellow fever infection in adult mice. *Nature.* **216**:179.
17. Hirsch, M. S., F. A. Murphy, A. J. Nahmias, and H. S. Kaye. 1968. Anti-thymocyte serum effects on viral infections. *Federation Proc.* **27**:734.
18. Allison, A. C., L. D. Berman, and R. H. Levey. 1967. Increased tumour induction by adenovirus type 12 in thymectomized mice and mice treated with anti-lymphocyte serum. *Nature.* **215**:185.
19. Allison, A. C., and L. W. Law. 1968. Effects of anti-lymphocyte serum on Moloney virus leukemogenesis and polyoma virus oncogenesis. *Proc. Soc. Exptl. Biol. Med.* **127**:207.
20. Hirsch, M. S., and F. A. Murphy. 1968. Effects of anti-thymocyte serum on Rauscher virus infection of mice. *Nature.* **218**:478.
21. Boyle, J. J., R. F. Haff, and R. C. Stewardt. 1967. Evaluation of antiviral compounds by suppression of tail lesions in vaccinia-infected mice. *In* Antimicrobial Agents and Chemotherapy. 1966. American Society for Microbiology, Ann Arbor. 536.
22. Glasgow, L. A. 1965. Leukocytes and interferon in the host response to viral infections: I. Mouse leukocytes and leukocyte-produced interferon in vaccinia virus infection in vitro. *J. Exptl. Med.* **121**:1001.
23. Fenner, F. 1958. The biological characters of several strains of vaccinia, cowpox, rabbitpox viruses. *Virology.* **5**:502.
24. Rabin, E. R., C. A. Phillips, A. B. Jenson, and J. L. Melnick. 1965. Vaccinia virus myocarditis in mice: an electron microscopic and virus assay study. *Exptl. Mol. Pathol.* **4**:98.
25. Montasir, M., E. R. Rabin, and C. A. Phillips. 1966. Vaccinia pneumonia in mice: a light and electron microscopic and viral assay study. *Am. J. Pathol.* **48**:877.
26. Fairbrother, R. W. 1933. The production of immunity to vaccinia virus by mixtures of immune serum and virus and the importance of phagocytosis in antivaccinial immunity. *J. Pathol. Bacteriol.* **36**:55.

27. Oppenheim, J. J. 1968. Relationship of in vitro lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Federation Proc.* **27**:21.
28. O'Connell, C. J., D. T. Karzon, A. L. Varron, M. C. Plautt, and V. M. Ali. 1964. Progressive vaccinia with normal antibodies: a case possibly due to deficient cellular immunity. *Ann. Internal Med.* **60**:282.
29. Hansson, O., S. G. O. Johansson, and B. Vahlquist. 1966. Vaccinia gangrenosa with normal humoral antibodies: a case possibly due to deficient cellular immunity treated with N-methyisatin B-thiosemicarbazone. *Acta Paediat. Scand.* **55**:264.
30. Baron, S., C. E. Buckler, R. M. Friedman, and V. McCloskey. 1966. Role of interferon during viremia: II. Protective action of circulating interferon. *J. Immunol.* **96**:17.
31. Schultze, H. E., and J. F. Heremans. 1966. Molecular Biology of Human Proteins: With Special Reference to Plasma Proteins. Elsevier, Amsterdam. **1**:732.
32. Russell, P. S. 1968. Antilymphocyte serum as an immunosuppressive agent. *Ann. Internal. Med.* **68**:483.
33. Woodruff, M. F. A. 1967. Anti-lymphocytic serum. *In* CIBA Foundation Study Group No. 29. Little, Brown and Company, Boston. 108.

Sections stained with hematoxylin and eosin.

FIG. 3. Section of tail from a mouse inoculated intraperitoneally with anti-thymocyte serum and intravenously with vaccinia virus. Dermal necrosis and acute inflammatory infiltrate. × 500.

FIG. 4. Lung from mouse inoculated intraperitoneally with anti-thymocyte serum and intravenously with vaccinia virus. Focal coagulative necrosis and cellular debris. × 150.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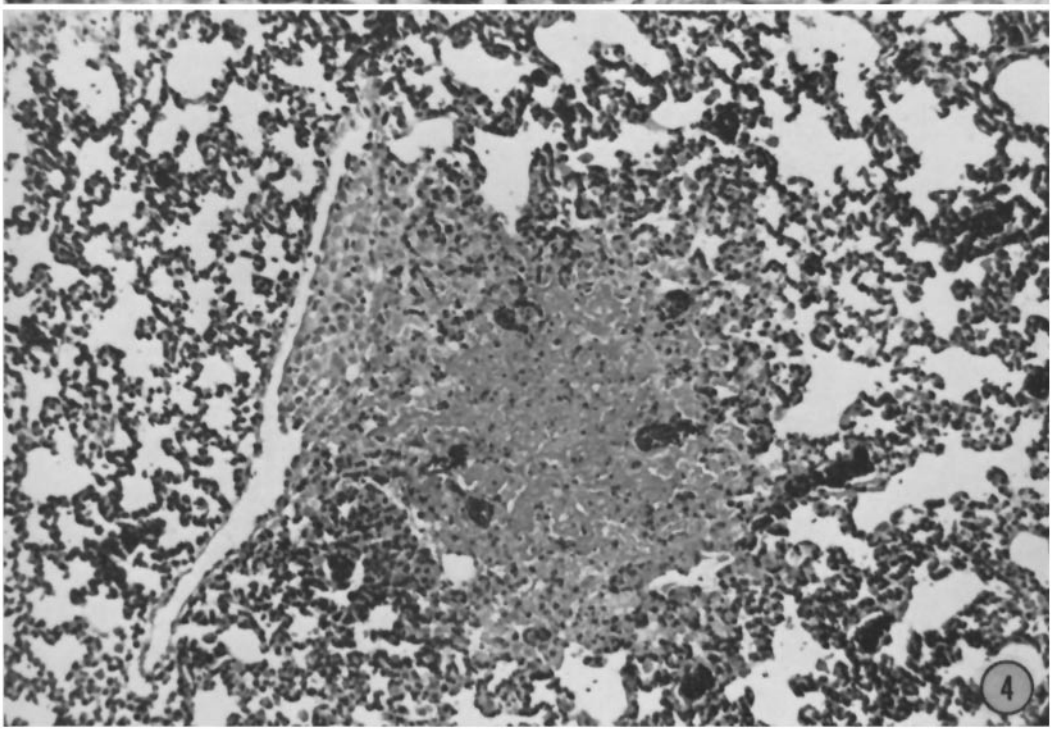
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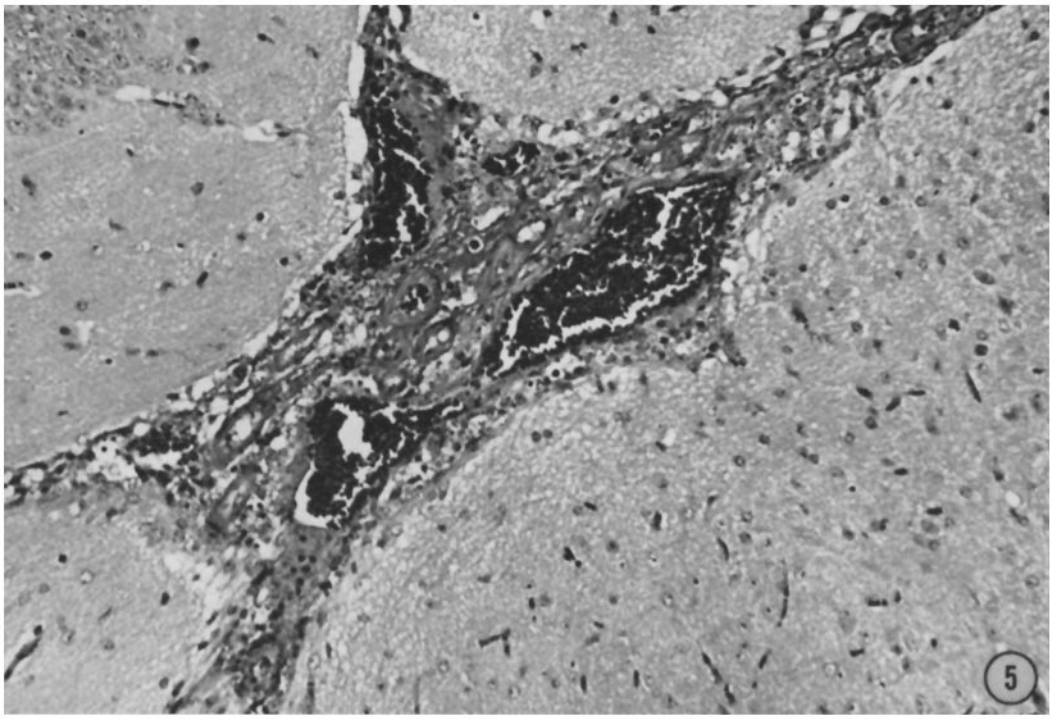

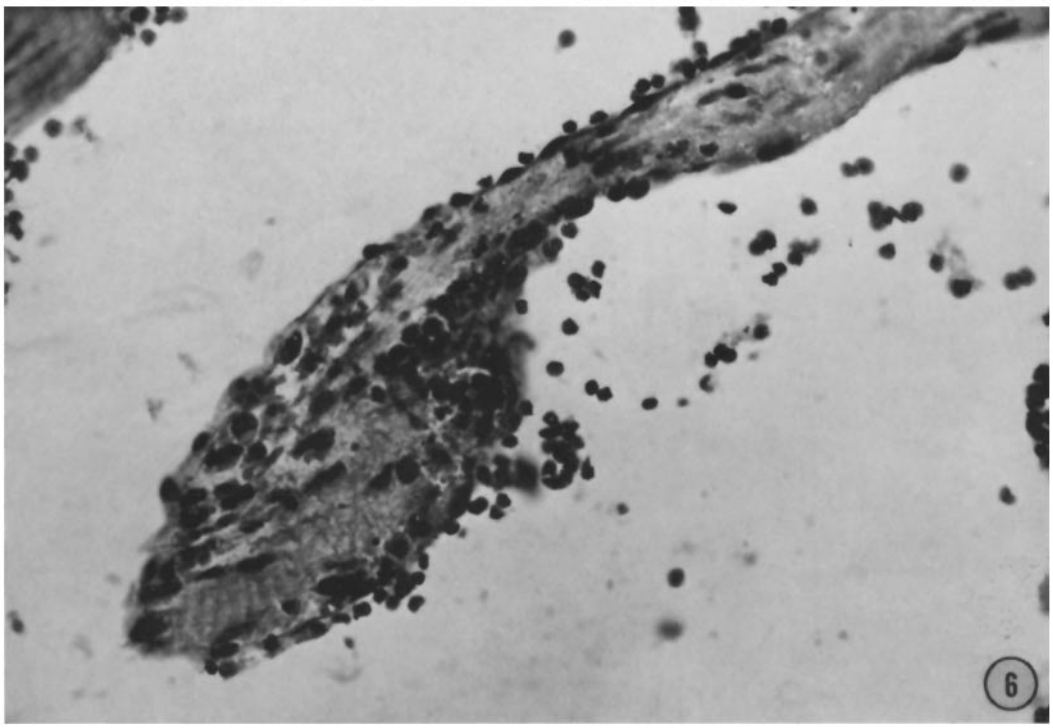

FIG. 5. Meninges from mouse inoculated intraperitoneally with anti-thymocyte serum and intravenously with vaccinia virus. Acute inflammatory exudate, congestion, and necrosis. × 180.

FIG. 6. Mitral valve from mouse inoculated intraperitoneally with anti-thymocyte serum and intravenously with vaccinia virus. Inflammatory infiltrate of polymorphonuclear leukocytes and plasma cells as higher magnification made plain. × 500.