INFECTION AND IMMUNITY, Jan. 1982, p. 151–156
0019-9567/82/010151-06$02.00/0

Vol. 35, No. 1

Permissiveness of Rabbit Monocytes and Macrophages for Herpes Simplex Virus Type 1

SUSAN PLAEGER-MARSHALL, LAWRENCE A. WILSON, AND JERRY W. SMITH*

Department of Microbiology and Immunology, Louisiana State University Medical Center, New Orleans, Louisiana 70112

Received 29 June 1981/Accepted 11 September 1981

The permissiveness of rabbit monocytes and macrophages for herpes simplex virus was examined. Peripheral blood monocytes, alveolar macrophages, and peritoneal exudate macrophages were studied for their ability to replicate herpes simplex virus strains RE and KOS. Results indicated different degrees of interaction with virus depending on the macrophage type. Only peritoneal exudate macrophages showed evidence of virus replication. Productive infection was limited, with only a small number of cells (0.02%) yielding infectious virus. Higher numbers of cells appeared to be abortively infected. Approximately 40% expressed antigens, whereas virtually all were killed by exposure to virus. Coreless particles were seen by electron microscopy in about one-third. Alveolar macrophages were also killed by virus and showed evidence of virus adsorption, but showed no indication of productive or abortive infection. Monocytes neither adsorbed nor replicated virus, and viability was unaffected. Results suggest that differences in degrees of cellular maturation or differentiation, or both, account for the spectrum of interactions seen between herpes simplex virus and rabbit macrophages.

Macrophages play a significant role in the establishment and spread of several viral diseases, including infections caused by herpes simplex virus (reviewed in 13, 17, 30). The ability of macrophages to permit virus replication has emerged as an in vitro correlate of the increased severity of herpetic disease in young mice (7, 9, 15, 16, 32). Efforts to expand studies of macrophage permissiveness to adult animals and to humans, however, have sometimes yielded contradictory results (3, 11, 12, 25). The ability of macrophages to replicate HSV appears to be critically affected by experimental conditions, including the strain of mouse used, strain of virus tested, multiplicity of virus used to infect, and the level of differentiation of the macrophage itself (3, 11, 12, 21, 25, 29).

Laboratory animals other than the mouse have received little attention regarding HSV-macrophage interactions. Although the mouse has provided a good system for macrophage studies, work has been limited to peritoneal cells because of the difficulty in obtaining macrophages from other sources. Another model used to study herpetic disease, particularly disease of the eye, is the New Zealand white rabbit. The rabbit offers three readily available sources of macrophages: blood (peripheral blood monocytes); lung (alveolar macrophages); and peritoneum (peritoneal exudate macrophages). Use of these cells allows a comparative study of macrophages from the same animal at various levels of differentiation (4, 19, 30). The purpose of this study was to determine the extent of permissiveness of each cell type for infection with HSV.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: HSV, herpes simplex virus; PBM, peripheral blood monocytes; AM, alveolar macrophages; PEM, peritoneal exudate macrophages; MOI, multiplicity of infection; HBSS, Hanks balanced salt solution without calcium or magnesium.

Virus. The KOS and RE strains of HSV type 1 were used. KOS, originally isolated from a case of herpes labialis, is a common laboratory strain (22) and was used by us previously in a study of the permissiveness of human leukocytes for HSV type 1 (21). The RE strain, isolated from a human corneal lesion, has been used extensively in studies of herpetic eye disease in rabbits (8). Both stocks were prepared from supernatant fluids from infected Vero cells. Monolayers were infected at an MOI of 0.1, and supernatants were harvested after 2 to 3 days at 34°C, when almost total cytopathic effect was evident. Fluids were clarified by centrifugation, and the titers were determined on Vero cell monolayers (28).

For viability experiments, virus was inactivated by either heat or UV light. Heat inactivation was accomplished by incubation of virus in a water bath at 56°C for 1 h. Virus was UV inactivated by exposure to a germicidal light (GE 15T8) for 30 min at a distance of 6 cm.

Animals. Adult New Zealand white rabbits weighing

2 to 3 kg were used. All were negative for serum antibody to HSV, as determined by plaque neutralization.

Macrophages. PBM were obtained by centrifugation of blood on Ficoll-Hypaque gradients (21). Briefly, animals were anesthetized by intravenous injection of 50 mg of sodium pentabarbitol and exsanguinated by cardiac puncture. Blood was mixed 5:1 with acid-citrate-dextrose to prevent coagulation and was diluted 1:4 with HBSS. The resulting mixture was centrifuged on a barrier gradient of Ficoll-Hypaque for 45 min at 500 × *g*. Mononuclear cells remaining at the interface were aspirated and washed three times in HBSS. Cells (2×10^5 to $4 \times 10^5/cm^2$) were incubated in plastic culture dishes at 37°C in 5% CO_2 for 2 h in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 1% glutamine (2 mM). Nonadherent cells were removed by washing with HBSS, and adherent cells were reincubated overnight in RPMI 1640 medium at 37°C in 5% CO_2. Adherent cells were >95% monocytes, as determined by morphology of Wright-stained cells and α-naphthyl acetate esterase activity (kit no. 90-A1, Sigma Chemical Co., St. Louis, Mo.). PBM were >98% viable, as determined by trypan blue exclusion.

AM were obtained by lavage of excised lungs by the method of Myrvik et al. (18). After overnight incubation in RPMI 1640 medium as described above, >98% of the adherent cells were macrophages, and virtually all were viable (>98%).

PEM were elicited by intraperitoneal injection of 35 ml of sterile paraffin oil 72 h before harvest. Cells were lavaged from the peritoneal cavity by infusion of 400 ml of cold HBSS through a midline incision. After three cycles of centrifugation to remove oil, cells were incubated in RPMI 1640 medium overnight. Adherent populations contained >98% macrophages and were essentially all viable (>98%).

Virus replication. All cells were plated to achieve an adherent population of approximately 5×10^5 cells in 1 ml of RPMI 1640 medium in 16-mm (approximately 2 cm^2) wells of a 24-well plastic plate. Virus was diluted in medium to achieve MOIs of approximately 100, 10, 1, and 0.1. Virus suspensions were added to wells in 0.3-ml amounts, and adsorption was allowed to proceed for 2 h at 37°C in 5% CO_2. Unadsorbed virus was removed by washing three times with 1 ml of HBSS, and fresh RPMI 1640 medium was added. Cells and fluids from triplicate wells were harvested at designated times postinfection and stored at −70°C. Plaque titrations were done with the use of Vero cell monolayers (28).

Infectious center assay. Cells were seeded in 24-well plates and incubated in the presence of virus as described above. After 2 h, unadsorbed virus was neutralized by treatment for 30 min with a 1:10 dilution of HSV antiserum produced in rabbits (27). Monolayers were washed to remove unreacted antibody, and cells were gently scraped from the wells with the edge of a siliconized rubber slab (Rodheim-Reiss, Inc., Belle Mead, N.J.). After counting and viability determinations, cells from triplicate wells were seeded onto Vero cell monolayers in RPMI 1640 medium containing 0.9% agarose. Plaques were counted at 3 days, after a second overlay of agarose containing neutral red (28).

Electron microscopy. Approximately 10^7 cells were allowed to adhere to 60-mm (21-cm^2) plastic culture dishes and exposed to virus as described above. At 24 h postinfection, cells were washed with Tyrode's solution, pH 7.2, fixed in 1% glutaraldehyde, scraped from the dish, and pelleted by centrifugation (cells already detached were decanted, then washed and fixed after pelleting). Cell pellets were then postfixed in osmium tetroxide, dehydrated in an ethanol series, and embedded in epoxy resin (Araldite) according to standard procedures. Thin sections were stained with uranyl acetate and lead citrate, pH 7.0, and were examined in a Philips 201 electron microscope.

Immunofluorescence. Cells were plated and exposed to virus as described above in 24-well plates. At 6, 24, and 48 h, medium was removed and adherent cells were washed three times with phosphate-buffered saline, pH 7.0. Cells were scraped from the wells, counted, and applied to a glass microscope slide by centrifugation, by means of a Shandon Cytospin (Shandon Southern Instruments, Inc., Sewickley, Pa.). Slides were air dried, fixed for 10 min in cold (−20°C) acetone, and drained dry. Staining was done with a 1:10 dilution of fluorescein-conjugated rabbit anti-HSV type 1, MacIntyre stain (Flow Laboratories, Inc., McLean, Va.).

Virus adsorption. After 24 h of incubation, medium was removed and virus was added to the wells at 5×10^3 PFU in 50 μl. At 15, 30, and 60 min, 1 ml of RPMI 1640 medium was added to the wells, and the supernatants were aspirated and pooled. After centrifugation to remove any cells that had detached, titers of fluids were determined in Vero cell monolayers to determine the amount of unadsorbed virus (28).

RESULTS

Virus replication curves. In initial experiments, macrophages were examined for replication of both virus strains KOS and RE. An MOI of 10 was used, based on previous work (3, 5, 21). Although Vero cells used as permissive controls showed increases in virus titer of approximately 2 logs, the macrophages examined showed no evidence of productive infection (Figure 1A and B).

To determine whether the MOI of virus used might have masked low levels of virus production or caused generation of defective interfering particles (24), we used a range of MOIs. Neither AM nor PBM showed rises in titer at MOIs of 100, 10, 1, or 0.1 with either virus strain. PEM also showed no increases in titer at MOIs of 100 or 10. Slight increases in yield, however, were seen when PEM were exposed to MOIs of 1 or 0.1 (Fig. 2).

Infectious center assay. Because production of infectious virus by macrophages appeared to be low or absent when a direct assay of virus replication was used, the more sensitive indirect technique of infectious center production was used. By this method, alveolar cells and monocytes were still negative for virus production. Experiments with PEM, however, showed that

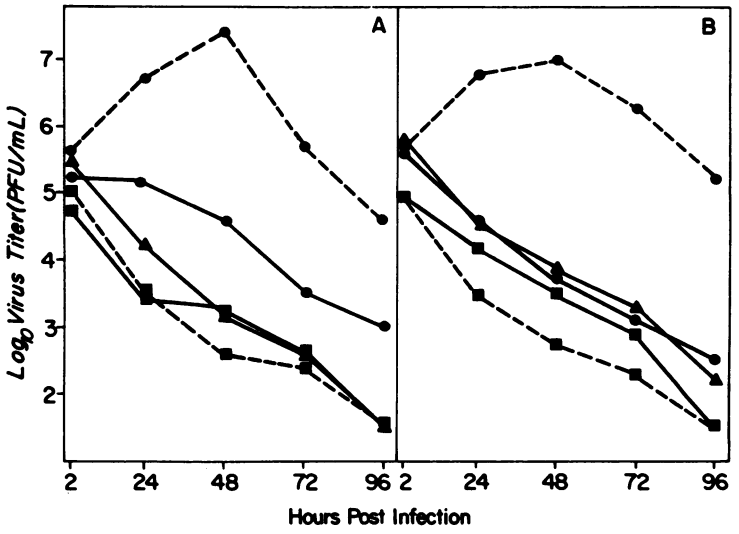

FIG. 1. Replication of HSV type 1 strains KOS (A) and RE (B) in rabbit macrophages. Cells were infected at an MOI of 10 PFU/cell. Symbols: ●—●, PEM; ■—■, AM; ▲—▲, PBM; ●--● Vero control; ■--■ virus inactivation control.

about 0.02% of the cells exposed to virus produced infectious particles (Table 1). Results were consistent at all four MOIs tested and with both virus strains.

Electron microscopy. To determine whether abortive replication occurred in macrophages, we examined cells by transmission electron microscopy. Whereas AM and PBM were negative for intranuclear virus particles, about 34% of the PEM were positive (Table 1). The particles seen generally lacked the central dense cores of complete virions, a characteristic indicative of abortive infection (3, 29).

Production of viral antigen. Fluorescent-antibody experiments were done to determine whether abortive infection could have resulted in production of viral antigen without evidence of virion formation observable by electron microscopy. By 6 h, approximately 38% of the peritoneal cells were fluorescent compared with uninfected controls (Table 1). Cells tested at 24 and 48 h showed evidence of extensive degeneration and could not be interpreted. Both AM and PBM were negative for fluorescence.

Viability of cells exposed to virus. Viability studies were done to determine whether abortive infection of PEM resulted in cell death. AM and PBM were also examined to determine whether killing occurred in the absence of replication.

To minimize manipulations that might further damage fragile infected cells (1), we developed a technique for examining adherent cells without removal from the wells. Macrophages were exposed to virus for 2 h, and unadsorbed virus was removed by washing three times. A micrometer (10 by 10 mm) was inserted into the eyepiece of an inverted microscope (American Optical Corp., Buffalo, N.Y.) and aligned with marks made on each well bottom. An area 10 mm square was counted at a magnification of 100× (approximately 1,000 cells), and the plate was reincubated. Preliminary experiments indicated

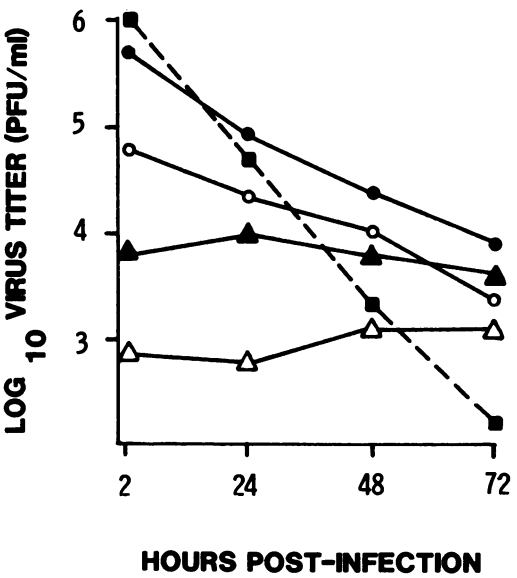

FIG. 2. Replication of HSV type 1 strain RE in PEM at four MOIs. Symbols: ●—●, 100 PFU/cell; ○—○, 10 PFU/cell; ▲—▲, 1 PFU/cell; △—△, 0.1 PFU/cell; ■--■, virus inactivation control.

TABLE 1. Percentage of PEM showing evidence of infection with HSV

Evidence of infection	No. of rabbits	Reactive cells (%)[a]
Viral antigen (immunofluorescence)	4	38 ± 2
Virus particles (electron microscopy)	5	34 ± 8
Infectious virus (infectious center assay)	5	0.02 ± .002

[a] Values represent mean ± standard error × 100.

that virtually all adherent cells were viable immediately after virus exposure. At 48 h, the same areas of the wells were recounted. Results of previous viability determinations showed that detached cells were dead, whereas the cells remaining adherent were still viable (data not shown). The number of adherent cells remaining at 48 h was therefore a function of viability and provided the basis for the viability assays.

Both PEM and AM showed some degree of virus-induced death. The cytocidal effect was dose dependent and was exerted by infectious and not inactivated virus. Some killing was detectable by 6 h after exposure to virus, with maximal death by 48 hr. PBM were unaffected by exposure to any MOI (Table 2).

Virus adsorption. Adsorption of HSV to the macrophages was studied to determine whether the failure of virus to exert an effect on monocytes was due to inability of these cells to adsorb virus. Figure 3 shows that, by 60 min, both PEM and AM adsorbed about 40% of the initial inoculum, and Vero control cells adsorbed more than 50%. Monocytes, however, adsorbed less than 10%, a value consistent with the nonspecific adsorption seen in wells containing no cells.

DISCUSSION

The data presented suggest that, in the New Zealand white rabbit, macrophage differentiation is the factor determining permissiveness for HSV. Based on previous reports, the three macrophage types used in our study probably represent various stages of maturation or differentiation, or both.

Each stage has been shown to differ in morphology, enzyme content and localization, phagocytic and metabolic properties (4, 19), and cytotoxic activities (A. M. Sheppard and J. W. Smith, manuscript submitted). Blood monocytes of the rabbit, like those of other species, appear to represent a cell of maturity intermediate between monocytic bone marrow cells and those macrophages found in tissues. PEM, on the other hand, are derived directly from blood monocytes, but possess a greater degree of functional activity than do PBM. AM appear also to derive from monocytes, but demonstrate unique oxidative functions consistent with their site of localization and have greater enzymatic properties than do PEM (4, 19, 30).

Of the three cells, PEM demonstrated the most extensive interaction with HSV. An increase in virus titer was observable only at low virus input multiplicities, with higher MOIs masking virus produced by a small number of productively infected cells. The apparent decreases in yield seen at high MOIs appeared not to be the result of generation of defective interfering virus particles since numbers of infectious centers were similar at all MOIs. Results were similar with both strains of HSV, suggesting that the findings were properties of the cells and not due to virulence factors associated with a particular virus strain. Since it is virtually impossible to obtain preparations of macrophages with 100% purity, a contaminating permissive cell type could have been responsible for the small number of productively infected cells. Although complete virions were seen by electron microscopy in cells appearing to be PEM, the possibility of contamination cannot be ruled out.

Replication of HSV within PEM could not completely account for the degree of cell death seen in viability experiments. Cell death was dose dependent and was mediated only by infectious virus. A similar "toxic" effect on macrophages has been reported to occur after exposure to vaccinia virus (26). Much like HSV-mediated killing, the cell death seen with vaccinia virus occurred early after exposure to a high MOI, even in the absence of viral replication. Release of internal virus proteins into the cell cytoplasm with subsequent alteration of membrane permeability has been suggested as a mechanism of cell killing by vaccinia virus (26).

TABLE 2. Percent viability of infected cells[a]

Cell type	Virus input multiplicity					Inactivated virus
	100	10	1.0	0.1	0	
PEM	4.2 ± 0.8	8.4 ± 1.2	43 ± 1.9	57 ± 3	95 ± 0.5	93 ± 1.5
AM	22 ± 2	48 ± 1.4	92 ± 0.8	94 ± 0.5	93 ± 0.5	94 ± 0.3
PBM	96 ± 0.4	95 ± 0.3	96 ± 0.3	97 ± 0.8	95 ± 0.2	94 ± 0.6

[a] Values represent mean ± standard error × 100. Cells were examined at 48 h postinfection.

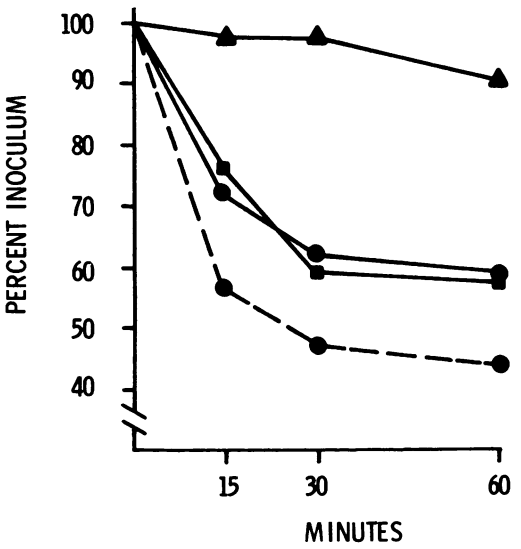

FIG. 3. Adsorption of HSV type 1 strain RE by rabbit macrophages. Values represent the percentage of the original inoculum in the supernatant fluid. Symbols: ●—●, PEM; ■—■, AM; ▲—▲, PBM; ●--●, Vero cell control.

Our data showed that at least three levels of interaction occurred between HSV and PEM. These findings are not too surprising in view of the heterogeneity of the oil-induced population in the rabbit. At least five subpopulations of PEM have been identified by gradient separation and found to differ in morphology, antigen binding, and number and activity of Fc receptors (10, 23, 31). Although the reason for the heterogeneity of elicited peritoneal macrophages is not known, the most plausible explanation appears to be differences in cellular maturation or differentiation, or both, within the PEM population (10, 31).

AM showed less extensive interaction with HSV than did PEM. Up to 80% of AM could be killed by exposure to a high MOI of virus, possibly by a mechanism similar to that responsible for the death of PEM. The failure of AM to support replication of HSV indicates species differences in the permissiveness of lung macrophages, since human AM produce small amounts of infectious virus (5, 15).

Monocytes, the least differentiated cells examined, showed no detectable interaction with HSV. The data suggest that rabbit PBM differ from human monocytes, which show abortive replication of HSV (3, 21).

The concept that macrophage differentiation may be critical to permissiveness of viral replication appears to extend to other viruses and animal systems. Whereas oil-elicited PEM from normal rabbits have been shown to support productive replication of vaccinia virus, PEM from animals immunized with vaccinia virus showed only abortive replication (2, 14). Restriction was attributed to macrophage activation resulting from infection, with the characteristic cellular differentiation occurring during the activation process (20).

Use of different mouse strains and HSV strains by various workers has resulted in reports of both permissive and abortive replication in mouse peritoneal cells (9, 12, 25, 29). The work of Lopez and Dudas (12), however, indicated that the apparent variations have resulted more from differences in methods used to obtain the cells than from genetic- or virus strain-related factors. Within a given mouse strain, elicited peritoneal cells were found to differ from resident cells, whereas freshly isolated cells differed from cultured cells. Human monocytes have been similarly shown to change in permissiveness for HSV when cultured (3, 11, 21). Cultured PBM are known to develop properties much like those of cells obtained from human peritoneal effusions (6).

The data obtained with the New Zealand white rabbit extend findings obtained from examination of cells from mice and humans. Macrophages, a heterogeneous group of cells displaying wide variation in morphology and function, exhibit heterogeneity in their interactions with HSV. The key molecular mechanism(s) governing the ability of these cells to replicate virus remains to be elucidated, but appears to relate to the level of their differentiation.

LITERATURE CITED

1. **Adams, D. O.** 1979. Macrophages. Methods Enzymol. **58**:494–506.
2. **Buchmeier, N. A., S. R. Gee, F. A. Murphy, and W. E. Rawls.** 1979. Abortive replication of vaccinia virus in activated rabbit macrophages. Infect. Immun. **26**:328–338.
3. **Daniels, C. A., E. S. Kleinerman, and R. Snyderman.** 1978. Abortive and productive infections of human mononuclear phagocytes by type 1 herpes simplex virus. Am. J. Pathol. **9**:119–129.
4. **Dannenberg, A. M., Jr., M. S. Burstone, P. C. Walter, and J. W. Kinsley.** 1963. A histochemical study of phagocytic and enzymatic functions of rabbit mononuclear and polymorphonuclear exudate cells and alveolar macrophages. J. Cell Biol. **17**:465–486.
5. **Drew, W. L., L. Mintz, R. Hoo, and T. N. Finley.** 1979. Growth of herpes simplex and cytomegalovirus in cultured human alveolar macrophages. Am. Rev. Resp. Dis. **119**:287–291.
6. **Hammerstrom, J.** 1979. Human macrophage differentiation *in vivo* and *in vitro*. Acta Pathol. Microbiol. Scand. **87**:113–120.
7. **Hirsch, M. S., B. Zisman, and A. C. Allison.** 1970. Macrophages and age-dependent resistance to herpes simplex virus in mice. J. Immunol. **104**:1160–1165.
8. **Irvine, H. R., and S. J. Kimura.** 1967. Experimental stromal herpes simplex keratitis in rabbits. Trans. Am. Ophthalmol. Soc. **65**:189–210.

9. **Johnson, R. T.** 1964. The pathogenesis of herpes virus encephalitis. II. A cellular basis for the development of resistance with age. J. Exp. Med. **120**:359–374.
10. **Kavai, M., J. Laczko, and B. Csaba.** 1979. Functional heterogeneity of macrophages. Immunology **36**:729–732.
11. **Linnavuori, K., and T. Hovi.** 1981. Herpes simplex virus infection in human monocyte cultures: dose-dependent inhibition of monocyte differentiation resulting in abortive infection. J. Gen. Virol. **52**:381–385.
12. **Lopez, C., and G. Dudas.** 1979. Replication of herpes simplex virus type 1 in macrophages from resistant and susceptible mice. Infect. Immun. **23**:432–437.
13. **McGeorge, M. B., and P. S. Morahan.** 1978. Comparison of various macrophage-inhibitory agents on vaginal and systemic herpes simplex virus type 2 infections. Infect. Immun. **22**:623–626.
14. **McLaren, C., H. Cheng, D. L. Spicer, and W. F. Tompkins.** 1976. Lymphocyte and macrophage responses after vaccinia virus infections. Infect. Immun. **14**:1014–1021.
15. **Mintz, L., W. L. Drew, R. Hoo, and T. N. Finley.** 1980. Age-dependent resistance of human alveolar macrophages to herpes simplex virus. Infect. Immun. **38**:417–420.
16. **Mogensen, S. C.** 1978. Macrophages and age-dependent resistance to hepatitis induced by herpes simplex virus type 2 in mice. Infect. Immun. **19**:46–50.
17. **Mogensen, S. C.** 1979. Role of macrophages in natural resistance to virus infections. Microbiol. Rev. **43**:1–26.
18. **Myrvik, Q. N., E. S. Leake, and B. Fariss.** 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. J. Immunol. **86**:128–132.
19. **Nichols, B. A., D. F. Bainton, and M. G. Farquhar.** 1971. Differentiation of monocytes: origin, nature, and fate of their azurophil granules. J. Cell Biol. **50**:498–515.
20. **North, R. J.** 1978. The concept of the activated macrophage. J. Immunol. **121**:806–809.
21. **Plaeger-Marshall, S., and J. W. Smith.** 1978. Experimental infection of subpopulations of human peripheral blood leukocytes by herpes simplex virus. Proc. Soc. Exp. Biol. Med. **158**:263–268.
22. **Rawls, W. E., D. Laurel, J. L. Melnick, J. M. Glickman, and R. H. Kaufman.** 1968. A search for viruses in smegma, premalignant and early malignant cervical tissues. The isolation of herpes virus with distinct antigenic properties. Am. J. Epidemiol. **87**:647–655.
23. **Rice, S. G., and M. Fishman.** 1974. Functional and morphological heterogeneity among rabbit peritoneal macrophages. Cell. Immunol. **11**:130–145.
24. **Schroder, C. H., and G. Urbaczka.** 1978. Excess of interfering particles over infectious particles in herpes simplex virus passaged at high M.O.I. and their effect on single-cell survival. J. Gen. Virol. **41**:493–501.
25. **Sethi, K. K., and H. Brandis.** 1979. In vitro acquisition of resistance against herpes simplex virus by permissive murine macrophages. Arch. Virol. **59**:157–172.
26. **Silverstein, S.** 1970. Macrophages and viral immunity. Semin. Hematol. **7**:185–214.
27. **Smith, J. W., and J. C. Glorioso.** 1976. Effect of cross immunization on monotypic antibody responses to herpes simplex virus types 1 and 2. J. Immunol. **116**:898–903.
28. **Smith, J. W., J. E. Rodriguez, and A. P. McKee.** 1971. Biological characteristics of cloned populations of herpes simplex virus types 1 and 2. Appl. Microbiol. **21**:350–357.
29. **Stevens, J. G., and M. L. Cook.** 1971. Restriction of herpes simplex virus by macrophages. An analysis of the cell-virus interaction. J. Exp. Med. **133**:19–38.
30. **VanFurth, R.** 1979. Cells of the mononuclear phagocyte system. Nomenclature in terms of sites and conditions, p. 1–30. *In* R. VanFurth and Z. Cohn (ed.), Mononuclear phagocytes. N. V. Nyhoff, Boston.
31. **Walker, W. S.** 1976. Functional heterogeneity of macrophages in the induction and expression of acquired immunity. RES J. Reticuloendothel. Soc. **20**:57–65.
32. **Zisman, B., M. S. Hirsch, and A. C. Allison.** 1970. Selective effects of anti-macrophage serum, silica, and anti-lymphocyte serum on pathogenesis of herpes virus infection of young adult mice. J. Immunol. **104**:1155–1159.