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Binding and Internalization of Herpes Simplex Virus–Antibody Complexes by Polymorphonuclear Leukocytes

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We studied the interactions between rabbit polymorphonuclear leukocytes (PMN) and the RE strain of herpes simplex virus type 1 (HSV-1) to determine better the role of inflammatory cells in herpetic stromal keratitis. PMN were found to be nonpermissive for HSV replication and were unable to bind virus in the absence of antibody. However, PMN did bind and internalize HSV–antibody complexes in vitro as was demonstrated visually by electron microscopic studies and quantitatively by measurement of activity associated with radiolabeled HSV–antibody complexes. Virus used for immune complex formation was labeled with either ^{125}Iodine or ^{35}S-methionine. In some experiments, anti-HSV IgG used for immune complex formation was labeled with ^{125}Iodine before incubation with virus. Use of all three radiolabeling approaches resulted in the same general pattern of binding, indicating a requirement for both antibody and virus for interaction with PMN. The activity associated with PMN was increased by preincubation with complement. The results suggest an active role for PMN in controlling HSV infection through their ability to bind and ingest virus–antibody complexes.

Key words: herpes simplex virus type 1, polymorphonuclear leukocyte, antigen–antibody complex

INTRODUCTION

Traditionally, the most important cells involved in antiviral immunity have been thought to be those comprising the lymphocyte–macrophage defense system. Although polymorphonuclear leukocytes (PMN) have been shown to function as primary effector cells in acute inflammatory responses to bacterial and mycotic infections, there is increasing evidence to suggest PMN play an important role in antiviral defense.

PMN have been observed in the inflammatory infiltrates in specific infections; for example, the cerebrospinal fluid of children with viral meningitis [Feigin and

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Shakelford, 1973] and the cutaneous lesions of varicella zoster infections [Stevens et al, 1975]. In herpetic stromal keratitis, a disease characterized by corneal opacity and scarring, an infiltrate consisting mainly of PMN and macrophages persists in the limbus and later the cornea for 4–5 days postinfection [Metcalf and Reichert, 1979].

A possible role for PMN in lower respiratory tract infections caused by respiratory syncytial virus (RSV) has been suggested by demonstrating in vitro activation of oxidative and arachidonic acid metabolism of PMN with RSV immune complexes [Kaul et al, 1981; Faden et al, 1983].

Although interactions that occur between host phagocytes and herpes simplex virus (HSV) during infection have not been extensively investigated, it has been assumed that the known mechanisms of antimicrobial immunity mediated by PMN participate in the activation and clearance of virus and virus–antibody complexes [Notkins et al, 1970; Theofilopoulos and Dixon, 1979; Sedlacek, 1980]. Previous studies in our laboratory, using chemiluminescence as an indicator of membrane perturbation and/or phagocytosis, suggest that PMN exert anti-HSV activity in the presence of specific antibody, but the virus alone has no effect [Bingham et al, 1985]. In an effort to clarify further the interactions between PMN and HSV in ocular infection, we studied the ability of PMN to mediate clearance of virus and virus–antibody complexes. The rabbit was used for our studies because the clinical disease in this model parallels the sequence of disease in human herpes keratitis [Meyers-Elliot et al, 1983]. The parameters addressed were: (1) the permissiveness of PMN to HSV replication, (2) the ability of PMN to bind and ingest HSV, and (3) the ability of PMN to bind and phagocytize HSV–antibody complexes.

MATERIALS AND METHODS

Preparation of PMN

Blood was collected from adult New Zealand white rabbits into a syringe containing heparin (20 units/ml blood). PMN were separated from whole blood by modification of the method described by Boyum [1968]. Briefly, 10 ml of heparinized blood was diluted 1:4 with Hanks balanced salt solution (HBSS), layered on a Ficoll–Hypaque gradient, and centrifuged for 40 min at 400 g. The resulting erythrocyte–PMN pellet was resuspended in 20 ml of autologous plasma and 8 ml of a 4.5% Dextran T-500 solution and incubated for 60 min at 37°C.

Following incubation, the PMN-rich supernatant was collected and spun for 10 min at 300 g. Residual erythrocytes were lysed with ACK buffer (30 mM NH_4Cl, 2 mM $KHCO_3$, 0.02 mM disodium EDTA). PMN purity was greater than 95% as assessed by Wrights stain and PMN viability exceeded 90% as determined by trypan blue exclusion.

Preparation of Virus

The RE strain of HSV-1 was used. This strain was originally isolated from a human corneal lesion and has been used in studies of herpetic stromal disease in rabbits without a high incidence of mortality from encephalitis. It produces a spectrum of disease in rabbits comparable to that seen in humans [Irvine and Kimura, 1967; Metcalf et al, 1976; Wander et al, 1980].

The RE strain was grown in SIRC (Staaten Serum Institut Rabbit Cornea) monolayers with minimal essential medium (MEM) and 5% fetal calf serum (FCS) at

37°C. When cytopathological changes involved 100% of the monolayer, culture fluid from infected cells was clarified by two cycles of low speed centrifugation (500 g for 15 min). The resulting supernatant was precipitated with polyethylene glycol (PEG) as described by Powell and Watson [1975] to obtain an enriched population of enveloped virus.

After an overnight incubation, the HSV precipitate was layered over a continuous 20–60% sucrose gradient and centrifuged at 85,000 g for 60 min. The virus band was collected, repelleted, rebanded on a sucrose gradient, and resuspended in MEM supplemented with 2% FCS and stored at −70°C. The PEG-purified HSV preparation had a titer of 8×10^7 plaque forming units (PFU)/ml in Vero cells.

Virus Replication in PMN

Peripheral blood PMN from normal rabbits were suspended in RPMI-1640 medium with 20% rabbit serum and seeded into 24-well tissue culture plates (5×10^5 PMN/well). After 60 min at 37°C, PMN cultures were incubated with HSV until harvest or exposed to virus 2 hr, washed, and overlaid with fresh medium. Culture supernatants were harvested at various time intervals up to 96 hr. Fluids were clarified by centrifugation (500 g for 10 min) and quantitated for plaque forming units on Vero cell monolayers using 1% agarose in MEM as the overlay medium.

Virus Adsorption to PMN

Both rabbit peripheral blood PMN and Vero cells were adjusted to a concentration of 2×10^6 cells/ml in RPMI-1640 supplemented with 10% rabbit serum. We added 0.5 ml of cells or medium alone to wells of a 24-well plastic tissue culture plate and mixed with 0.1 ml of HSV at 22°C. Virus was diluted to achieve input multiplicities of infection (MOI) of 10 and 1. At designated times after addition of virus (5, 15, 30, and 60 min) culture fluid was harvested, clarified by centrifugation (500 g for 10 min), and quantitated by plaque assay in Vero cells.

Purification of IgG

Antiserum to the RE strain of HSV-1 was produced in New Zealand white rabbits weighing 2–3 kg. Each eye was injected intrastromally with 0.02 ml of virus preparation containing 10^6 PFU.

At 28 days postinfection, animals were exsanguinated and the blood allowed to clot at room temperature. The serum was separated by one cycle of centrifugation and then heated for 30 min at 56°C to inactivate complement.

IgG was precipitated from immune serum with a neutralization titer of 1024 by the addition of ammonium sulfate and then separated from other charged serum proteins by ion exchange chromatography on DEAE cellulose [Garvey et al, 1977]. Peak protein fractions from the column were pooled and concentrated on a PM-10 membrane (Amicon Corp., Lexington, MA). The protein content of purified IgG was determined by absorbance at 280 nm and purity analysis done by Ouchterlony immunodiffusion.

Radiolabeling of Purified HSV and IgG

Purified HSV was radiolabeled with ^{125}Iodine (New England Nuclear, Boston, MA) either by the lactoperoxidase method described by Fenger et al [1978] or through

metabolic incorporation of the amino acid ^{35}S-methionine [Eberle and Courtney, 1980].

Purified IgG was radiolabeled by the chloramine-T method of McConahey and Dixon [1966] and by the lactoperoxidase technique of Marchalonis [1969], modified by Kozel and McGaw [1979].

Preparation of Virus–Antibody Complexes

Purified radioiodinated HSV (1.3 mg/ml protein) was combined in equal parts with either a 1:10 dilution of rabbit antiserum to HSV or commercial rabbit serum that showed no antibody to HSV. Following a 30-min incubation at 37°C, virus–antibody complexes and free virus were pelleted by centrifugation (85,000 g for 60 min), washed twice with MEM plus 2% FCS, and stored at −70°C. Immune complexes containing ^{35}S-HSV were made, washed, and stored in a similar manner.

Radioiodinated IgG (1 mg/ml) was prepared from rabbit antiserum to HSV and combined in equal volumes with purified virus. The mixture was incubated for 30 min at 37°C in a shaking waterbath and then centrifuged at 1000 g for 30 min to sediment the insoluble complexes. The complexes were washed twice, resuspended in MEM plus 2% FCS, and stored at −70°C.

Immune Complex Binding Assay

The uptake of radiolabeled complexes by rabbit PMN was measured by a modification of the coverslip assay of Smith and Rommel [1977]. In this assay system, 12 mm round #1 thickness coverslips were cleaned with acetone and placed in 16 mm wells of a 24-well tissue culture plate. PMN in RPMI-1640 supplemented with 20% normal rabbit serum were seeded onto the coverslip (5×10^5 PMN/coverslip) and allowed to adhere for 1 hr at 37°C in a 5% CO_2 atmosphere. Control (blank) coverslips were treated with medium plus rabbit serum. Following incubation, each coverslip was washed with medium to elute nonadherent cells and serum components.

We added 50 μl of radiolabeled complexes to each coverslip. After a 10-min incubation at 37°C, the coverslips were removed from wells, washed four times with medium, drained dry with a tissue, and inserted into a bio-vial (Beckman). Iodinated samples were counted on a Biogamma counter (Beckman) to quantitate coverslip associated radioactivity. Quantitation of ^{35}S-radioactivity was accomplished by tissue solubilization and liquid scintillation spectrometry as described by Smith et al [1979].

Electron Microscopy

Complexes formed by combining PEG concentrated HSV and ammonium sulfate precipitated IgG were incubated with rabbit PMN for 5 min at 37°C. In preparation for thin sectioning, PMN were washed twice with Tyrode's solution (pH 7.2), fixed in 1% glutaraldehyde, postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin (Araldite). Thin sections were stained with uranyl acetate and lead citrate (pH 7.0) and examined in a Phillips 201 electron microscope.

RESULTS

Permissiveness of Rabbit Peripheral Blood PMN to HSV Replication

Studies were done to determine if the interaction between HSV and PMN results in viral replication. The findings in Figure 1 indicate that no detectable replication of

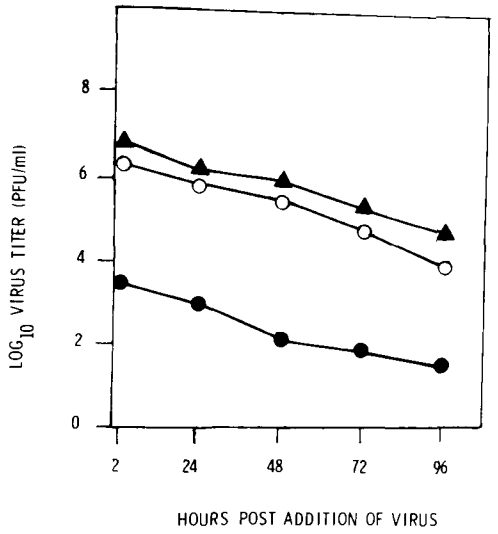

Fig. 1. Replication of HSV in PMN. PMN from peripheral blood of normal rabbits were allowed to adhere to bottoms of 24-well plastic tissue culture plates (5×10^5 cells/well) before exposure to infectious virus (MOI of 100). Tissue culture fluid was harvested at various times after addition of virus. The titer of infectious HSV was determined by plaque assay under agarose on Vero cell monolayers. Results are expressed as the mean of triplicate cultures. Symbols: ▲ medium + HSV; ● PMN + HSV incubated for 2 hr at 37°C and washed to remove HSV inoculum; ○ PMN + HSV incubated together at 37°C.

HSV occurs in rabbit PMN. Cultures exposed to infectious virus for 2 hr or longer showed no increase in virus yield. Slight decreases in virus titer were observed in all samples examined beyond 2 hr. Both PMN exposed to virus and that incubated with medium alone exhibited similar patterns of loss of infectivity. Electron microscopy revealed no evidence of permissive or abortive infection in PMN examined 24–48 hr after addition of HSV (data not shown).

Binding of HSV to Rabbit Peripheral Blood PMN

To determine if rabbit PMN bound or absorbed HSV, experiments were performed in which infectious virus was exposed to PMN in culture. Supernatant fluids were examined for virus at various time intervals. Results shown in Figure 2 indicate no adsorption or binding of HSV to PMN. Supernatant fluids contained the same amount of infectious virus before and after incubation with PMN for both MOI's examined, 10 and 1. Vero cell monolayers served as positive binding controls. Decreases in virus titer were observed in supernatants from Vero cells as early as 5 min after exposure to HSV.

Binding of HSV–Antibody Complexes to PMN Using Radiolabeled Virus Preparations

In an effort to determine if virus–antibody complexes interact with PMN, experiments were performed in which enveloped HSV, either externally labeled with ^{125}Iodine or metabolically labeled with ^{35}S-methionine, was exposed to immune serum and the resulting complexes were assayed for binding. Results in Table I show that enveloped ^{125}I-HSV incubated with normal rabbit serum was bound to PMN at

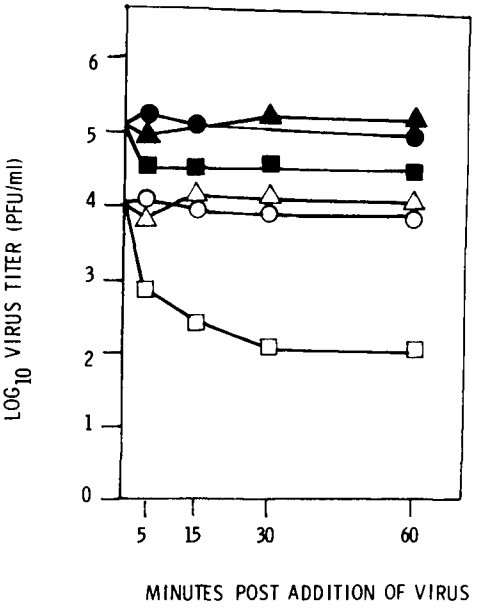

Fig. 2. Binding of HSV to PMN. Infectious HSV was added to suspensions of either 2×10^6 rabbit PMN or 2×10^6 Vero cells at MOI's of 10 and 1. The cultures were continuously agitated and at designated time intervals aliquots were collected. Culture fluids were clarified of cells by centrifugation at 500 μg and screened for reduction in HSV titer by plaque assay under agarose on Vero cells. Results are expressed as mean values of triplicate samples. MOI of 10: ▲ medium + HSV; ● PMN + HSV; ■ Vero + HSV. MOI of 1: △ medium + HSV; ○ PMN + HSV; □ Vero + HSV.

TABLE I. Binding of Virus-Antibody Complexes to PMN Using Radiolabeled HSV Preparations

Reaction mixture		Activity
Cells	Complex	(CPM)[a]
None	^{125}I-HSV + NRS[b]	980 ± 82
None	^{125}I-HSV + Ab	1446 ± 162
PMN	^{125}I-HSV + NRS	4591 ± 171
PMN	^{125}I-HSV + Ab	14942 ± 1001
None	^{35}S-HSV + NRS	105 ± 10
None	^{35}S-HSV + Ab	602 ± 54
PMN	^{35}S-HSV + NRS	2750 ± 244
PMN	^{35}S-HSV + Ab	6516 ± 595

[a]Results represent the mean of sextuplicate coverslips ± SEM.
[b]NRS, normal rabbit serum.

greater than fourfold the amount of activity associated with control coverslips. Addition of complexes of ^{125}I-HSV and HSV specific antibody produced activity tenfold greater than blank coverslips and threefold greater than that associated with PMN and virus exposed to nonimmune serum.

The same pattern of response was seen when ^{35}S-HSV was substituted for ^{125}I-HSV in immune complexes. Approximately the same ratios of activity were observed. Immune complexes formed using ^{35}S-HSV antibody demonstrated the highest activity associated with PMN.

Binding of Virus–Antibody Complexes to PMN Using Radiolabeled Anti-HSV IgG

The results of experiments using complexes comprised of ^{125}I-IgG and HSV to demonstrate binding to PMN are shown in Table II. With nonspecific binding controls (^{125}I-IgG and supernatant from uninfected SIRC cells), no increase in ^{125}I-IgG activity associated with PMN was detected above background binding levels. However, when complexes of ^{125}I-IgG and unlabeled HSV were combined with PMN, a tenfold increase in activity was observed.

Effect of Complement on the Binding of Virus–Antibody Complexes to PMN

To examine the effect of complement, experiments were performed in which either heat inactivated or native guinea pig complement was added to immune complexes. Incubation of immune complexes with native guinea pig complement produced an approximate twofold increase in activity bound to PMN over complexes incubated with heat inactivated complement (Table III). Controls showed an approximate fourfold increase in activity of ^{125}I-HSV and antibody complexes associated with PMN compared to background coverslips.

Internalization of HSV–Antibody Complexes by PMN

To determine whether the interactions between PMN and HSV–antibody complexes resulted in internalization by phagocytosis, we performed experiments utilizing

TABLE II. Binding of Virus–Antibody Complexes to PMN Using Radiolabeled Anti-HSV IgG

Reaction mixture		Activity
Cells	Complex	(CPM)[a]
None	^{125}I-IgG + medium[b]	274 ± 14
None	^{125}I-IgG + HSV[c]	339 ± 61
PMN	^{125}I-IgG + medium	342 ± 9
PMN	^{125}I-IgG + HSV	3419 ± 286

[a]Results represent the mean of triplicate coverslips ± SEM.
[b]Antibody incubated with supernatant from uninfected SIRC cultures (30 min at 37°C) and pelleted by ultracentrifugation (85,000 g for 60 min).
[c]Insoluble immune complexes pelleted by low speed centrifugation (1,000 g for 30 min) and washed prior to use.

TABLE III. Effect of Complement on the Binding of Virus–Antibody Complexes to PMN

Reaction mixture		Activity
Cells	Complex	(CPM)[a]
None	^{125}I-HSV + Ab + ΔC[b]	684 ± 101
None	^{125}I-HSV + Ab + C[c]	1166 ± 95
PMN	^{125}I-HSV + Ab + ΔC	2850 ± 230
PMN	^{125}I-HSV + Ab + C	4492 ± 304

[a]Results represent the mean of quadruplicate coverslips ± SEM.
[b]Complexes incubated for 30 min at 37°C with an equal volume of a 1:10 dilution of heat-inactivated guinea pig complement.
[c]Complexes incubated for 30 min at 37°C with an equal volume of a 1:10 dilution of guinea pig complement.

electron microscopy. Figure 3 shows the results of incubating virus–antibody complexes with PMN. Enveloped HSV can clearly be seen within phagocytic vacuoles and attached to the PMN cytoplasmic membrane. Experiments using HSV incubated either alone or after exposure to nonimmune serum resulted in no visible internalization of virions (data not shown).

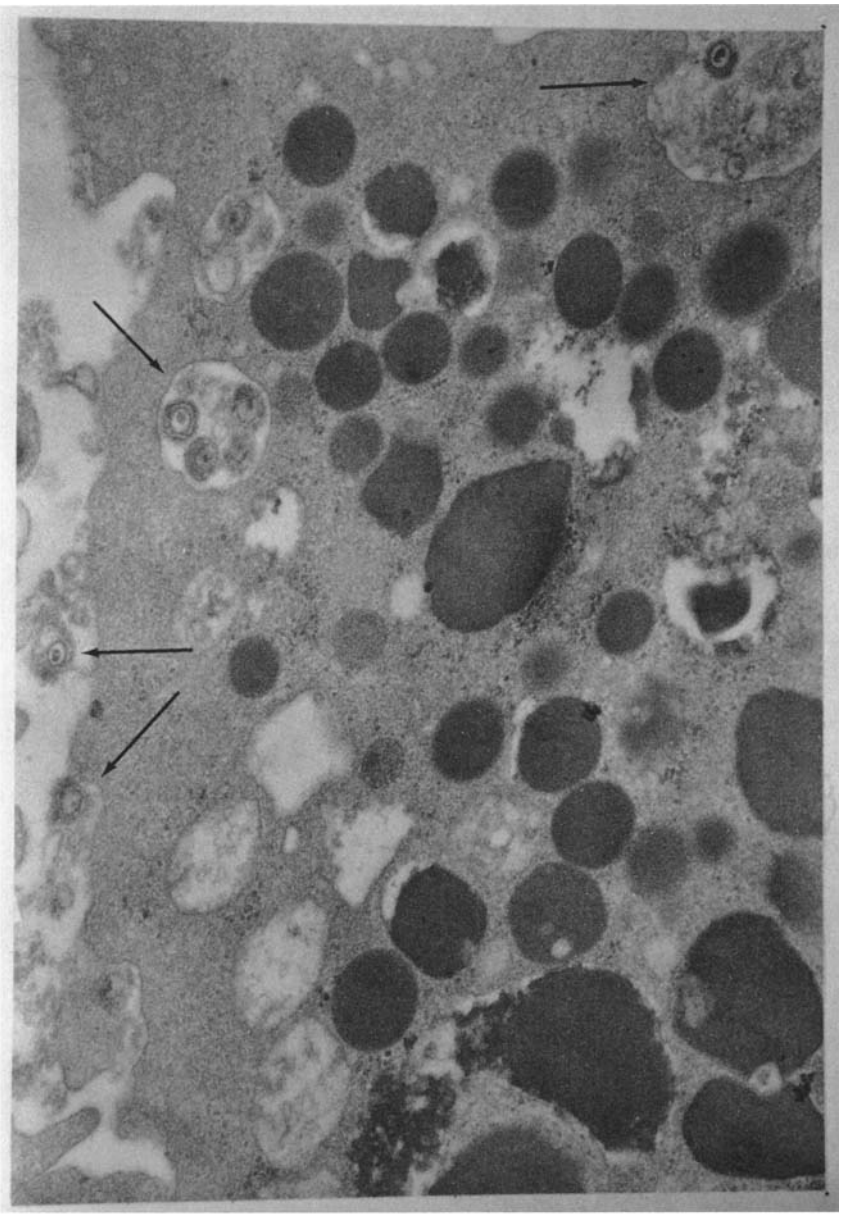

Fig. 3. Internalization of HSV–antibody complexes by PMN. The arrows denote complete enveloped HSV particles that are attached to the cytoplasmic membrane and internalized within the phagocytic vacuoles of rabbit PMN. Photograph was taken 5 min after addition of virus–antibody complexes to PMN. Magnification, ×36,000.

DISCUSSION

Herpetic stromal keratitis causes corneal damage that may result in blindness. At present, it is not clear whether PMN induce, mediate, or contribute to the pathogenesis of the disease in vivo.

Enhancement of HSV virulence may result from the ability of the virus to infect, replicate, and persist in phagocytic cells. Peritoneal exudate macrophages from strains of mice susceptible to HSV infection in vivo have been shown to support the replication of HSV [Brucher et al, 1984]. It has also been shown that HSV [Daniels et al, 1978], rubella [Van Deg Logt, 1980], and dengue-2 [Brandt et al, 1979] viruses are capable of replicating in human monocytes and macrophages. Our laboratory has reported HSV permissiveness following mitogenic stimulation of human lymphocytes [Plaeger-Marshall and Smith, 1978]. In addition, we have demonstrated limited permissiveness of rabbit macrophages to HSV infection [Plaeger-Marshall et al, 1981]. Rabbit monocytes, on the other hand, showed no detectable interaction with virus. In addition to in vitro studies, fluorescent antibody probes have suggested the presence of HSV or HSV antigens within PMN from infiltrates associated with herpetic keratitis [Meyers and Pettit, 1973; Meyers-Elliot et al, 1980; Meyers-Elliot and Chitjian, 1981].

In the present study of HSV-PMN interactions, no replication or adsorption of HSV to PMN was observed. Regarding replication, no increase in virus titer was seen in PMN exposed to HSV. Samples of PMN taken 24–48 hr after exposure to HSV and examined by electron microscopy showed no evidence of complete virions or abortive infection. Binding experiments using PMN mixed with HSV at two MOI's revealed no measurable adsorption, whereas Vero cell controls gave positive binding results under the same conditions. The data presented suggest that rabbit PMN do not recognize HSV by itself, supporting results from studies in this laboratory using chemiluminescence as an assay system [Bingham et al, 1985]. Explanations for PMN nonpermissiveness could be the phagocyte's lack of receptors, short life span, and limited capacity for protein synthesis, all of which my reflect differences in degrees of cellular maturation and differentiation.

It has been well established that PMN ingest and kill bacteria and fungi [Hilger and Danley, 1980; Klebanoff, 1975; Schuit, 1979]. In antiviral defense, PMN are presumed to be active in the clearance of virus-antibody complexes through phagocytosis following recognition by receptors that bind to antibody and complement [Allison, 1974]. Opsonins, such as IgG, interact with the material to be phagocytized, adding a host protein coating that facilitates internalization via the PMN Fc receptor [Eden et al, 1973; Phillips-Quagliata et al, 1969; Theofilopoulos et al, 1974; Wiggins and Cochrane, 1981].

A variety of techniques have been employed to quantitate phagocytosis by PMN. Some methods determine the number of particles removed from the culture medium; others count the number of particles that appear within phagocytes. The uptake of radioactive protein from the suspending medium is another approach to measuring phagocytosis [Chang, 1969]. Using this last approach, Ward and Zvaifler [1973] described a method for the quantitative measurement of phagocytosis by neutrophils using radiolabeled immune complexes. Because of particle size, virus uptake by cells cannot be visually assessed by light microscopy. Therefore, the ability to assay phagocytosis with labeled immune complexes becomes a particularly useful tool when studying the interactions between PMN and viruses.

In our current study using radiolabeled complexes, we have demonstrated that HSV-antibody complexes bind to PMN. We employed complexes containing either ^{125}I-HSV, ^{35}S-HSV, or ^{125}I-IgG and obtained similar patterns of response, thus minimizing the chance that the results observed were an artifact of assay conditions. Increased binding occurred using HSV labeled externally with ^{125}Iodine or metabolically with ^{35}S-methionine for the formation of antigen-antibody complexes. When radiolabeled antibody (^{125}I-IgG anti-HSV) was combined with virus in immune complexes, there was a dramatic increase in the binding activity to PMN in contrast to the low activity produced by unaggregated ^{125}Iodine labeled HSV-IgG controls. In an unrelated study, using similar procedures, Shinomaya and Koyama [1976] found that ^{125}I-IgG alone bound minimally to guinea pig macrophages whereas ^{125}I-IgG combined with hapten was rapidly bound and internalized.

The addition of complement to virus-antibody complexes is presumed to enhance neutralization and immune clearance through enlargement of viral aggregates. Studies have shown that immune complexes containing complement bind to phagocytes with greater affinity than those without complement [Kijlstra et al, 1979; Mantovani et al, 1972; Van Snick and Masson, 1978]. Our data support the concept that complement increases binding of immune complexes to PMN. The addition of active complement to radiolabeled complexes was found to increase activity associated with PMN by as much as 25%.

The data presented clearly demonstrate that PMN bind HSV-antibody complexes and that the binding activity increases with the addition of complement. To determine whether ingestion as well as adsorption of immune complexes occurs, we employed electron microscopy. This approach resulted in direct visualization of internalized HSV-antibody complexes. Enveloped virions were observed both within phagocytic vacuoles and adherent to the PMN cytoplasmic membrane.

In the initial stages of acute inflammation large numbers of PMN are recruited to destroy and remove the inflammatory stimuli via phagocytosis [Issekutz et al, 1981]. The presence of PMN in the corneal stroma during HSV keratitis can be explained by the liberation of proteases from damaged cells, the activation of the complement cascade by antigen-antibody complexes, the release of leukotactic factors by virus-sensitized lymphocytes, and the chemotactic properties of collagenase in the cornea [Meyers and Pettit, 1974].

Gradually, macrophages accumulate at the same locations for further removal of inflammatory stimuli. Macrophages also initiate tissue repair and immunological responses such as antigen presentation to T lymphocytes. Phagocytic cells at the sites of inflammation bind or ingest immune complexes formed during the course of the disease. Occasionally the removal of such complexes is not efficiently achieved resulting in the subsequent synthesis and release of more inflammatory mediators by PMN and macrophages [Davies and Bonney, 1981].

The pathogenesis of herpetic stromal keratitis remains unclear. It is not fully understood how much of the disease process is due to viral replication and how much is due to the immune response to viral antigens. Our observations that PMN are capable of binding and ingesting HSV-antibody complexes suggest that PMN play a beneficial role in the clearance of virus during herpetic keratitis.

Previous reports [Dawson and Togni, 1976; Pettit and Meyers-Elliot, 1973] have suggested a major role for specifically sensitized lymphocytes in chronic recurrent corneal inflammation. Our data do not exclude such a possibility. It is clear that

a combination of inflammatory cells are active during the course of herpetic keratitis and that a complete understanding of their interrelationships and mechanisms involved in immune destruction will require further investigation.

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