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REDUCED ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY TO HERPES SIMPLEX VIRUS-INFECTED CELLS OF SALIVARY POLYMORPHONUCLEAR LEUKOCYTES AND INHIBITION OF PERIPHERAL BLOOD POLYMORPHONUCLEAR LEUKOCYTE CYTOTOXICITY BY SALIVA[1]

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Blood polymorphonuclear leukocytes (BPMN) have been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) against HSV-infected cells. Although HSV infections are frequently found in the oral cavity, the ADCC capacity of salivary PMN (SPMN) has not been studied, mainly because methods to isolate SPMN were not available. We have recently developed a method to isolate SPMN, and in this study have evaluated their ADCC activity against HSV-infected cells. SPMN were obtained by repeated washings of the oral cavity, and separated from epithelial cells by nylon mesh filtration. ADCC was quantitatively determined by ^{51}Cr release from HSV-infected Chang liver cells. SPMN in the presence of antibody were able to destroy HSV-infected cells, but SPMN were much less effective in mediating ADCC than BPMN (3.4% vs 40.7%, $p < 0.0001$). In the presence of antiviral antibody, SPMN were able to adhere to HSV-infected cells, but less so than BPMN (34% vs 67%), and specific antibody-induced adherence was significantly lower in SPMN ($p < 0.04$). The spontaneous adherence to HSV-infected cells was higher for SPMN than BPMN. SPMN demonstrated up-regulation of the adhesion glycoprotein CD18, but down-regulation of the FcRIII receptor. Incubation with saliva decreased ADCC capacity of BPMN, up-regulated CD18 expression, and down-regulated FcRIII expression.

Human leukocytes have been shown to mediate ADCC[3] against HSV-infected cells (1). The elements of human ADCC were able to protect neonatal mice against lethal HSV-infection (2), and thereby may play an in vivo role in protection against HSV infections. The effector cell population in human peripheral blood has been shown to include killer lymphocytes (3), monocyte-macrophages (4), and BPMN (5–7). Although BPMN were able to mediate ADCC against HSV-infected cells, they were slower and required higher concentrations of immune serum than human mononuclear cells (5). Similar studies have demonstrated that colostral leukocytes could mediate ADCC against HSV-infected cells, although their activity was lower than BPMN, and required higher antibody concentration (8).

Although primary and recurrent HSV infection is frequent in the oral cavity (9), the ADCC capacity of SPMN has not been studied. The main reason has been lack of a method for separation of viable functional SPMN. Inasmuch as high numbers of PMN are present in the oral cavity (0.2 to 1.5×10^6/min, representing more than 98% of salivary white blood cells (10, 11), their capability to destroy HSV may play a significant role in protection of the oral mucosa against herpetic infection. We have developed a method to isolate functional SPMN based on their separation from oral epithelial cells by nylon mesh. We have demonstrated that they are stimulated cells showing high levels of H_2O_2 production, F-actin polymerization, and CD11b expression as compared to BPMN. Moreover, SPMN could significantly respond to further in vitro stimulus with increased H_2O_2 production and elevated F-actin levels (10).

The objectives of this study were to evaluate the ADCC capacity of SPMN in vitro, and compare their activity to BPMN from the same donor. Inasmuch as we observed reduced ADCC capacity of SPMN compared to BPMN, we determined their surface receptors expression (FcRIII and CD18) and also the effect of acellular saliva on the ADCC activity of BPMN.

MATERIALS AND METHODS

Blood and salivary PMN. Healthy volunteers, with mininal or no periodontal disease were tested. Blood and PMN were obtained from the same volunteers and tested simultaneously for ADCC capacity.

BPMN were obtained as described (5). Twenty ml of heparinized whole blood were mixed with 2 ml of 3% dextran and allowed to sediment (30 min, 37°C). The supernatant was removed and layered onto Ficoll-Hypaque (Pharmacia, Piscataway, NJ). The pellet containing PMN and red cells was then used. Virtually all the cells were shown to be viable when tested by trypan blue.

SPMN were obtained as described previously (10). Briefly, donors placed 15 ml of HBSS containing 0.1% gelatin without Ca or Mg ions in their mouth, and swished the solution. After 30 s, the HBSS was expectorated into a polypropylene vessel containing 400 ml of HBSS at 4°C. This procedure was repeated for 20 min. The expectorates were stirred for 10 min and spun for 10 min at $250 \times g$, resuspended in 50 ml HBSS, and passed sequentially through a 20-μm and 10-μm nylon mesh. The cells were then washed twice, and the viability

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[2] Address correspondence and reprint requests to Dr. Steve Kohl, Division of Pediatric Infectious Diseases, Department of Pediatrics, Room 6E7, San Francisco General Hospital, 1001 Potrero Ave., San Francisco, CA 94110.
[3] Abbreviations used in this paper: PMN, polymorphonuclear cells; BPMN, blood polymorphonuclear cells; ADCC, antibody-dependent cellular cytotoxicity; SPMN, salivary polymorphonuclear cells.

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of the cells was determined by trypan blue exclusion assay. About 90% of the cells were viable when tested by trypan blue. As previously reported, the purity of BPMN was 93%, with only 1% mononuclear cell contamination and 6% eosinophils (5), and the purity of SPMN was 99% (10).

Saliva. Saliva was obtained from two HSV-positive and two HSV-negative donors. Saliva was then vortexed, spun for 1 h at 500 × *g* at 4°C, and the supernatant was filtered through a 0.45-μm pore size filter. The acellular filtered saliva was dispensed into small volumes, and stored at −40°C until use.

Sera. Pooled immune sera was prepared from four individuals with prior HSV infections, as described (12). Pooled nonimmune sera was obtained from two individuals with no clinical history of HSV infection and no detectable titers of neutralizing or ADCC antibodies to HSV. All sera were heat-inactivated at 56°C for 30 min and stored at −40°C until use.

Microcytotoxicity assay. The microcytotoxicity assay was performed in triplicate in rigid polystyrene U-bottom microtiter plates (Cooke, Alexandria, VA) as described (12). Chang liver cells infected with HSV type 1 for 18 h, and labeled with ^{51}Cr were prepared as described previously (4). One hundred μl of effector cells (BPMN or SPMN, 5 × 10^6 cells/ml), 50 μl of target cells (Chang liver cells, 1 × 10^5 cells/ml), and 50 μl of serum diluted 1:5 were added to each well. The final serum concentration was 1:20, and the final E:T was 100:1. These concentrations were previously shown to promote maximal ADCC for PMN (5). The covered plates were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 for 18 h, unless otherwise specified. Cell lysis was determined by ^{51}Cr released from the target cells, as described (4). All samples were counted in a gamma counter (model 4000; Beckman Instruments, Palo Alto, CA) for 1 min. Percentage release of ^{51}Cr was calculated by the formula:

$$\frac{2A}{[A + B]} \times 100$$

where A is the cpm in the top μl and B is the cpm in the bottom 100 μl to which NaOH was added. The percentage ADCC was defined as:

$$\%\text{ADCC} = \frac{\left[\begin{matrix}\%^{51}\text{Cr release of target} \\ \text{cells + PMN + Immune sera}\end{matrix}\right] - \left[\begin{matrix}\%^{51}\text{Cr release of target} \\ \text{cells + PMN + nonimmune sera}\end{matrix}\right]}{\left[\begin{matrix}100 - \%^{51}\text{Cr release of target cells} \\ \text{+ PMN + nonimmune sera}\end{matrix}\right]} \times 100$$

The percentage ^{51}Cr release of target cells was 11.76% ± 3.9%, in the presence of immune or nonimmune sera. The SD of triplicate assays was generally less than 5%.

Pre-incubation of BPMN with saliva. Purified BPMN were preincubated with saliva for 5, 15, or 30 min, or with saliva diluted with HBSS (1%, 10%, and 100%) for 15 min. After incubation, the cells were washed twice using HBSS, counted, and their viability was determined by trypan blue exclusion. The final concentration of viable cells was determined.

Single cell adhesion assay. The single cell adhesion assay was performed by a modification of the single cell agarose assay (12). Briefly, nonradioactive HSV-infected Chang liver target cells (2 × 10^6 cells/ml, 50 μl) were incubated with 50 μl (2 × 10^6 cells/ml) of BPMN, SPMN, or BPMN incubated with saliva for 30 min (E:T of 1:1). Thirty-three μl of serum diluted 1:5 in MEM containing anti-HSV antibody was then added to the cells in 12 × 75-mm glass tubes. Thirty-three μl of serum without antibody diluted 1:5 was added to control groups. The suspension was then incubated for 5 min in a humidified incubator containing 95% air and 5% CO_2. After incubation, cells were centrifuged (250 × *g*), the supernatant was removed and the cell pellet was suspended in 50 μl of MEM. The pellet was resuspended 10 times with an Ependorf micropipet. Ten μl of the cell suspension were placed in a counting chamber and 200 leukocytes were counted to determine the number of leukocytes binding to target cells. Chang liver cells were easily distinguished from leukocyte effector cells by their larger size. Specific antibody adherence was calculated by the percentage of cells that adhered in the presence of serum containing HSV antibody minus the percentage of adherence in the presence of HSV negative serum.

Expression of FcRIII and CD18 receptors. FcRIII and CD18 receptors, the receptor for the Fc component of IgG (CD 16), and the β-subunit of the leukocyte adhesion glycoprotein complex, respectively, were determined by using mAb followed by staining with fluorescent secondary antibody, and were evaluated in flow cytometry (10). Ninety μl of cell suspension (BPMN, SPMN, or BPMN preincubated with saliva for 30 min in concentration of 10 × 10^6 cells/ml) were incubated (15 min, room temperature) with 10 μl of 3G8.10 SUP (mAb to the Ig receptor, FcRIII, kindly provided by Dr. Wayne Smith (13), or with 50 μl of TSI/18 ASC (mAb to the β-subunit of adhesive glycoproteins complex, CD18, kindly provided by Dr. Wayne Smith who originally obtained it from Timothy Springer, Center of Blood Research, Department of Pathology, Harvard Medical School, Boston, MA). Control cells were incubated with 10 μl of IgG1 (anti-mouse nonbinding antibody, Damon Biotech, Inc, Needham Heights, MA). After incubation, the cells were washed three times, resuspended in HBSS containing FITC-labeled rabbit anti-mouse IgG F(ab') fragment (Boehringer Mannheim Biochemicals, Indianapolis, IN), and were incubated for 15 min at room temperature in the dark. Subsequently, the cells were washed, lysed by using Becton Dickinson lysis solution (Becton Dickinson, Mountain View, CA), and resuspended in 1 ml of 5% paraformaldehyde. Cells were evaluated by using flow cytometry to determine the quantity of receptors expressed on the surface of the cells evaluated.

Samples of SPMN and BPMN were measured by using Becton Dickinson FACscan with a Hewlett Packard computer. Cells were illuminated by a 5-watt argon laser emitting 400 milliwatts of light at 488 nm. PMN analyzed for fluorescence were selected by using forward and right-angle light scatters as described previously (10). PMN were identified as having more right angle and forward scatters than lymphocytes and oral debris, but less forward scatter than epithelial cells. A green fluorescence histogram was collected for 5,000 to 10,000 PMN for each sample analyzed. The mean fluorescence given is the mean channel fluorescence of 100% of the cells that were selected by forward and right angle scatter. All the leukocytes selected expressed CD18 and FcRIII receptors.

Data analysis. Data are expressed as mean ± SEM. The significance of differences between BPMN and SPMN was determined by Student's *t*-test, whereas the differences between BPMN vs BPMN incubated with saliva were determined by using a paired *t*-test.

RESULTS

Comparison of salivary and blood PMN cell cytotoxicity. SPMN were able to mediate low levels of ADCC against HSV-infected cells. When compared to BPMN, their ADCC activity after 2, 4, and 18 h was significantly reduced (Fig. 1). The ADCC of BPMN rose higher and more rapidly than SPMN ADCC (Fig. 1). At 18 h, blood neutrophil ADCC was 40.7% ± 7.9, compared with the ADCC of SPMN of 3.4% ± 2.6 ($n = 6$ separate experiments, $p < 0.0001$). The ADCC activity of SPMN or BPMN from HSV positive or negative donors was similar. Similarly, the ADCC of cells from two donors with recurrent oral HSV infection was no different from those of the two seronegative donors and the seropositive donor with HSV recurrences.

The ability of SPMN and BPMN to mediate ADCC in the presence of increasing concentrations of antibody was compared (Fig. 2). Although BPMN were able to mediate ADCC at immune serum concentrations as low as 10^{-4}, SPMN did not mediate ADCC below immune serum dilutions of 10^{-2} (Fig. 2).

The effect of the separation technique of SPMN on ADCC of BPMN. Since SPMN had low ADCC, it was necessary to determine if the technique used to purify them was the cause of low cytotoxicity. The effect of the separation technique of SPMN on ADCC of BPMN was determined by washing the BPMN in HBSS (containing 0.1% gelatin, without Ca or Mg ions), passing them sequentially through 20- and 10-μm pore size nylon mesh, counting, and analyzing them for ADCC as described above. No significant change was found in the BPMN purified in this fashion as compared with controls (Fig. 3).

The effect of saliva on BPMN ADCC. To explain the profound defects in SPMN cellular cytotoxicity relative to the cellular cytotoxicity of matched BPMN, the effect of

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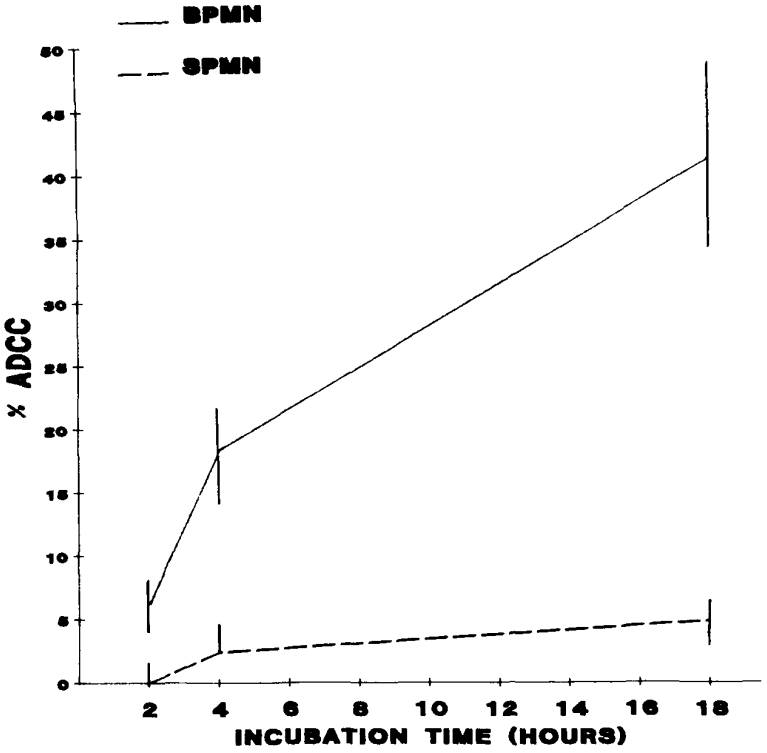


Figure 1. Kinetics of ADCC of blood and salivary neutrophils. The E:T was 100:1 for SPMN and BPMN. The antibody concentration was 1:20. The results are means ± SEM of two determinations done in triplicates.

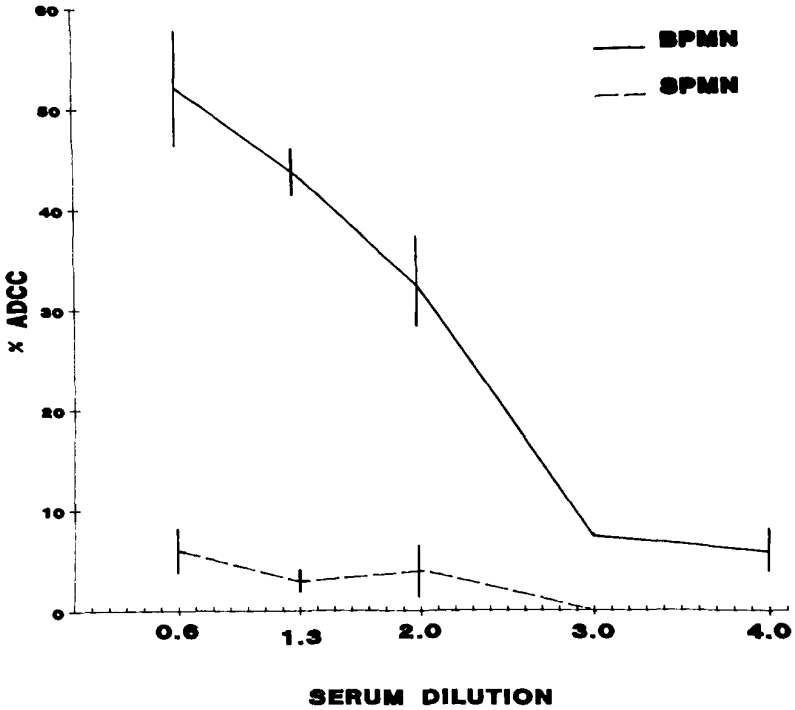


Figure 2. Antibody requirement for ADCC of blood and salivary neutrophils. The E:T was 100:1 and the incubation time was 18 h. Serum dilutions are presented as $\log_{10}$. The results are means ± SEM of two different experiments done in triplicates, except for the $10^{-1.3}$ dilution that represents mean of six different experiments done in triplicates.

acellular filtered saliva on the cytotoxicity of BPMN was analyzed. BPMN were incubated with saliva for 5, 15, or 30 min. Target cells and immune sera at concentrations previously shown to optimize ADCC were then added (an E:T of 100:1, and serum dilution of 1:20). For each time period tested, saliva inhibited ADCC activity of BPMN (Fig. 4). The inhibitory effect increased as the time of incubation increased. After 5-min incubation, saliva caused 39% inhibition, although the magnitude of inhibition was variable ($p > 0.2$; three experiments). After 15 min, saliva induced 56% inhibition, with less variability ($p < 0.006$; seven experiments), and the most profound inhibition was found after 30-min incubation (66%; $p = 0.057$; three experiments). Saliva diluted 1:10 and 1:100 did not induce significant inhibition of the ADCC of BPMN after 5- or 15-min incubation (Fig. 5). Incubation of SPMN in saliva (100%) for 30 min and then washing and incubating in media for 30 min failed to

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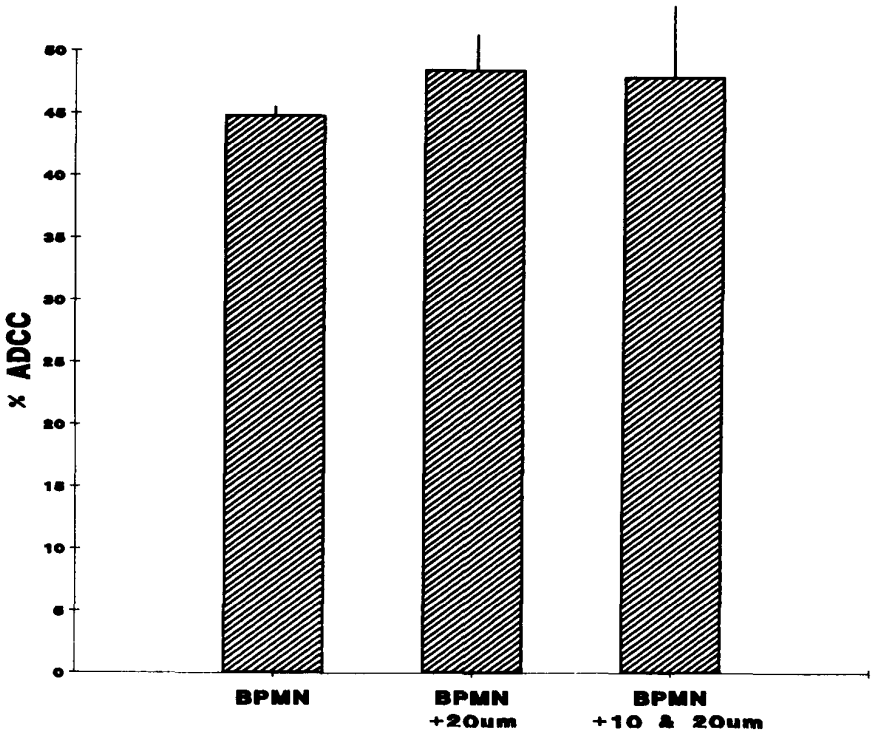


Figure 3. The effect of the separation technique of SPMN on ADCC of BPMN. After the SPMN separation procedure utilizing two pore sizes (20 μm or 10 + 20 μm), BPMN were analyzed for ADCC. The E:T was 100:1, and the serum dilution was 1:20. The results represent the mean ± SEM of two different experiments done in triplicates.

regenerate ADCC (control 41%, saliva and media incubated 0%). Similarly, incubation of BPMN in saliva that had been previously heated to 100°C for 5 min or 60°C for 30 min failed to reverse the inhibitory effect of 100% saliva (0% ADCC).

To determine whether the HSV serology of saliva was important in the inhibitory effect of BPMN on ADCC to HSV, the effect of saliva from seropositive and seronegative donors was analyzed. There was no significant difference ($p = 0.42$) in the inhibition of ADCC of BPMN by saliva obtained from seropositive or seronegative donors (data not shown).

Single cell adhesion of BPMN, SPMN, and BPMN incubated with saliva. To further analyze possible mechanisms responsible for the defect seen in SPMN, the ability of BPMN, SPMN, and BPMN incubated with saliva (30 min) to adhere to HSV-infected target cells was measured in a single cell adhesion assay (Fig. 6). BPMN, SPMN and BPMN incubated with saliva bound significantly more to target cells in the presence of anti-HSV antibody (67% ± 8, 34% ± 6, and 51% ± 2, respectively), than in the absence of antibody (3% ± 0.1, 10% ± 4, and 4% ± 1, respectively). BPMN had a significantly higher specific antibody-mediated adherence as compared to SPMN (64 vs 24%, $p < 0.04$). BPMN incubated with saliva had a lower specific binding as compared with BPMN (47 vs 64%). In contrast, the nonspecific binding (in the absence of anti-HSV antibody) was higher but not significantly for SPMN (10% ± 4) as compared to BPMN or to BPMN incubated with saliva (3 and 4%, respectively).

The effect of saliva on the expression of FcRIII and CD18 receptors. BPMN, SPMN, and BPMN incubated with saliva for 30 min, were evaluated for the expression of IgG receptor-FcRIII, and the β-component of the leukocyte adhesion glycoprotein complex receptor-CD18, using FACS (Becton Dickinson) (Fig. 7). CD18 expression by SPMN was 2.75-fold higher than that of BPMN. Incubation of BPMN with saliva increased the expression of CD18 of BPMN by 94%. In contrast, SPMN FcRIII receptor expression was only 17% of BPMN. BPMN incubated with saliva had a 56% decrease in the expression of FcRIII receptor as compared to control BPMN. In four separate experiments, the mean fluorescence of cells incubated with anti-FcRIII mAb was 1015 ± 242 for BPMN, 175 ± 49 for SPMN, and 570 ± 293 for BPMN incubated in saliva. The significance of these differences are $p < 0.04$ for BPMN compared to BPMN incubated in saliva, and $p < .0001$ for SPMN compared to BPMN. Mean fluorescence utilizing the control mAb was minimal (BPMN 14, SPMN 45, and BPMN incubated in saliva 12).

DISCUSSION

The results of this study showed that SPMN were able to mediate low levels of ADCC against HSV-infected cells. SPMN had significantly lower activity than BPMN, although they were 90% viable. The SPMN ADCC capacity was markedly lower than that of PMN of most other sources studied to date, such as BPMN of adults or infants, when similar E/T are used (5, 7, 8, 14, 15). The only PMN that have shown similarly low ADCC capacity are those derived from patients with LFA-1/OKM-1 (CD11/18) deficiency (12). We have previously demonstrated by 24-h incubation experiments (which ablate PMN-ADCC, but not lymphocyte ADCC), and lymphocyte addition experiments that BPMN ADCC is due to PMN, not contaminating lymphocyte effector cell function (5).

The mechanism of ADCC involves leukocyte target recognition, binding, lysis, and recycle (12, 16–20). The reasons for the reduced ADCC capacity of SPMN are not completely clear. Several defective steps in the ADCC

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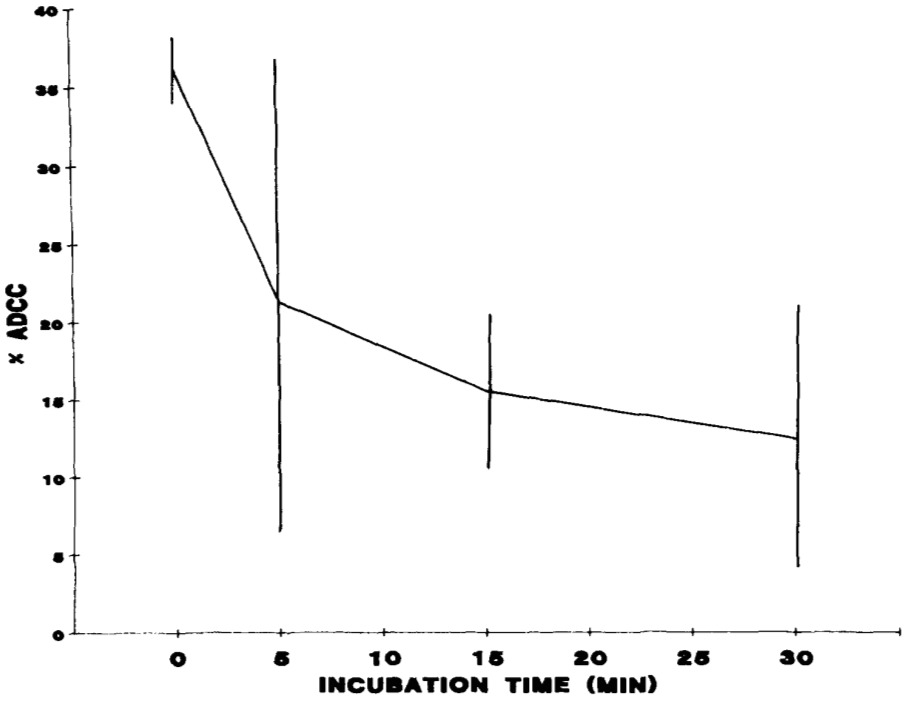


Figure 4. Saliva inhibition of ADCC of BPMN. BPMN were incubated with saliva for 5, 15, or 30 min. The PMN were then washed twice, and tested for ADCC. The results (5 min, 30 min) represent the means ± SEM of three different experiments done in triplicates. The results (0 min, 15 min) represent the mean ± SEM of seven experiments done in triplicates.

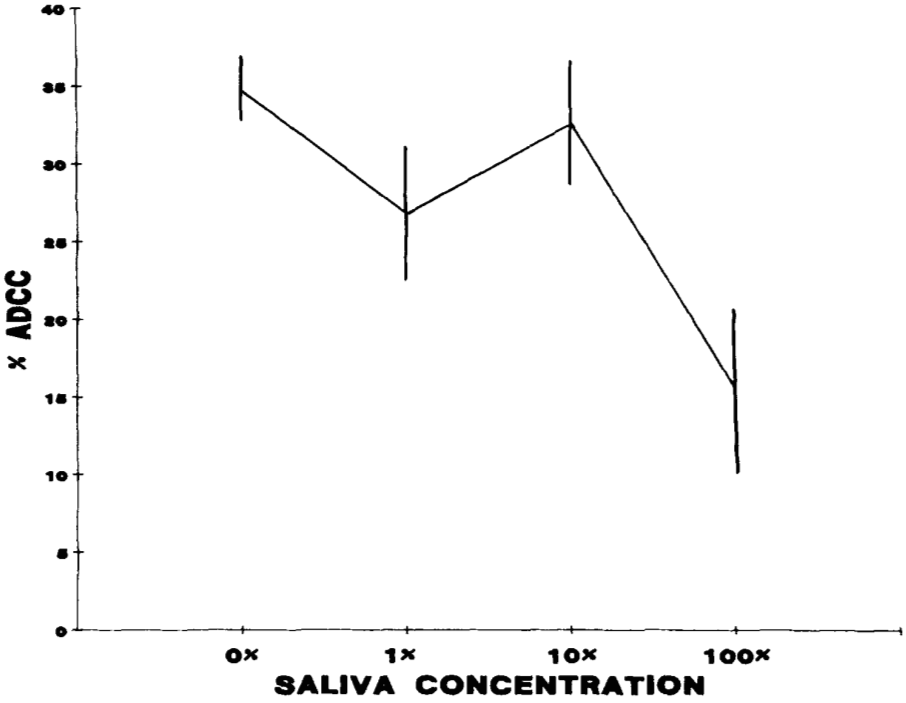


Figure 5. Titration curve of the inhibition of ADCC of BPMN by saliva. BPMN were incubated with different concentrations of saliva (1%, 10%, 100%) for 15 min, washed twice, and then were tested for ADCC. The E:T was 100:1, and the serum dilution was 1:20. The results (100%, 0%) represent the mean ± SEM of seven different experiments done in triplicates. The results (1%) represent the mean ± SEM of three different experiments done in triplicates. The results (10%) represent the mean ± SEM of four different experiments done in triplicates.

process may be involved. The initial prerequisite step in ADCC is recognition of the antibody-sensitized infected cells by the Fc receptors. We examined the expression of FcRIII receptors in SPMN, and have found profound decreases (84%) of this receptor as compared with BPMN. The decreased Fc receptor expression in SPMN could be a result of masking, enzymatic degradation, or phagocytosis with subsequent internalization of Fc receptors on PMN, as occurs with latex or colostral phagocytosis (4, 14, 21). PMN, unlike macrophages, cannot renew their plasma membrane (22). Decreased Fc receptor expression has been reported also following stimulation with phorbol ester-PMA, although to a lesser degree (23, 24).

The second step in ADCC is binding to the infected target cells (17–19). This step is mediated at least in part by the leukocyte adhesion glycoproteins (CD11/18). We have examined the expression of CD18 in SPMN, and have found that SPMNs expressed a marked increase (175%) in their CD18 receptor expression. CD18 is the β-component of the adhesion glycoprotein complex and is the common component of three different adhesion glycoprotein receptors (LFA-1 or CD11a/CD18, MAC-1 or CD11b/CD18, P150, 95 or CD11c/CD18). These receptors differ by their α-component, the CD11a, CD11b, and

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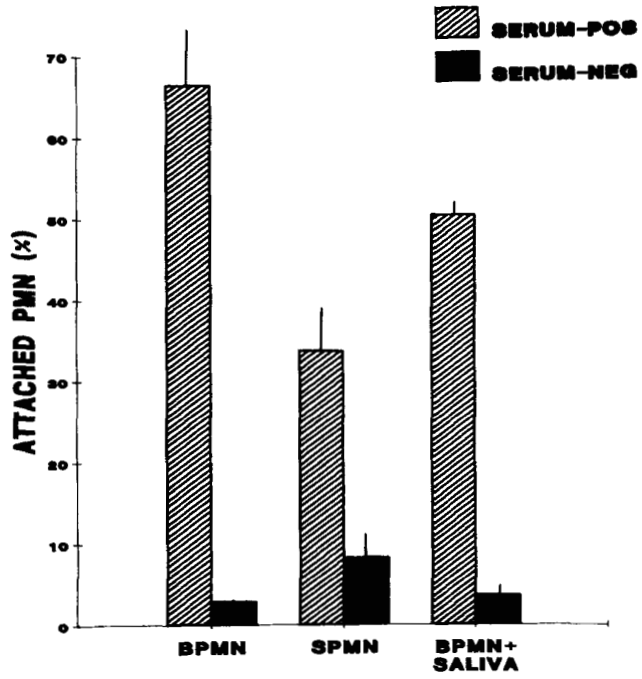


Figure 6. Single cell adhesion assay of BPMN, SPMN, and BPMN incubated with saliva, to HSV-infected cells. The results are the percentage of PMN binding target cells after 5-min incubation, in the presence of 1:20 dilution of anti-HSV antibody-containing serum (E:T of 1:1) and represent the mean ± SEM of two different experiments done in triplicates.

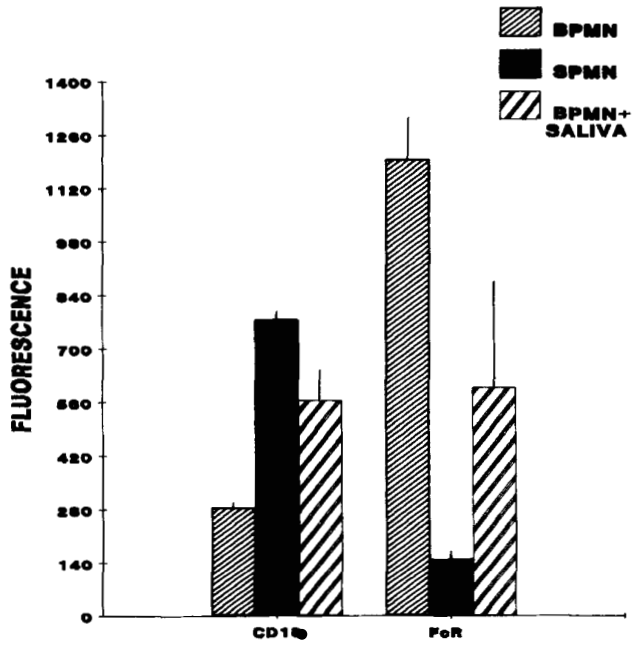


Figure 7. The expression of FcRIII and CD18 receptors on BPMN, SPMN, and on BPMN incubated with 100% acellular saliva for 30 min. After incubation with saliva, the BPMN were washed twice. The bars are the mean ± SEM of two separate experiments. The y-axis is mean fluorescence of gated PMN as described in *Materials and Methods*.

CD11c. The dramatic increase in the CD18 is in agreement with the finding that SPMN has 2 to 2.5 times increase in the α-component (CD11b) of the CD11b/CD18 receptor (10). The main component of the increase in the CD18 expression is related to the increase in CD11b/CD18. This might explain the increase in nonspecific binding of SPMN to HSV-infected cells, as well as the relatively high specific binding of the SPMN in the absence of normal FcR expression.

The third step in mediating ADCC is lysis of infected target cells (19–20). The Fc receptor is essential for this step, while the complement receptor may increase the lytic effect mediated by the Fc receptor. In SPMN, it seems that whereas high expression of CD11/CD18 can partially compensate for the low levels of Fc receptors in recognition and binding, it cannot compensate for decreased lysis of the infected target cells. This is supported by the finding that the interaction of virions through PMN-complement receptors CR1 and CR3 results solely in binding to PMN but not in internalization, whereas interaction via both complement and Fc receptors results in a very efficient internalization of viruses (25). The importance of intact Fc, as well as complement receptors, for intact ADCC capacity, is further supported by the very low ADCC capacity of BPMN from patients with CD11/18 deficiency (Mac-1 or CD11b/18 is the complement receptor C3bi) (12). These cells have normal Fc receptors but low or no adhesion glycoproteins, as opposed to SPMN which show low Fc receptor, but extremely high expression of CD11b/CD18.

The decreased ADCC capacity of SPMN is at least partially due to extrinsic effects of the saliva on cells. This was demonstrated by the profound inhibitory effect of acellular filtered saliva on purified BPMN ADCC and Fc receptor expression, after incubation of BPMN with saliva. Lower ADCC in SPMN could have also resulted from IgA antibody to HSV in saliva that might have competitively inhibited IgG-mediated ADCC by blocking target cell sites. If this occurred, saliva from seropositive donors should have been more inhibitory than that from seronegative donors. This was not the case. It remains possible that IgA (not specifically antiviral) may inhibit ADCC by interacting with Fc receptors on the effector cells. Other possible mechanisms of inhibition could involve the presence of enzymes or environmental toxins inhibitory to cells in saliva. In this regard, heat treatment of saliva failed to ablate its inhibitory effect of BPMN.

In conclusion, this study showed reduced ADCC capacity of SPMN associated with up-regulation of CD18 and down-regulation of FcRIII receptor. This reinforces the importance of intact Fc receptors regardless of the CD11/18 expression for ADCC function. We have also showed that SPMNs are functionally distinct from BPMN. It is unlikely that SPMN would have a role in recurrent oral HSV infection. The lesions typically arise at the mucocutaneous junction, and deep in the tissue where the virus is discharged from nerve endings. In contrast, primary oral HSV infection is a more superficial, intraoral infection, especially early in the course of infection. This is a time when ADCC antibody is first appearing. Indeed, it has been reported that the appearance of ADCC antibody to HSV in blood was associated with defervescence in children with primary HSV gingivostomatitis (26). The low effective activity of SPMN makes it unlikely that these cells are important ADCC effectors once they appear in saliva. It remains possible that these cells function in the oral tissue as ADCC effectors, or in the saliva in combination with IgA or complement as antiviral effector (25).

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