A Method for Quantitation of Human Leukocyte Adhesion to Glass[1] (36741)

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Adhesiveness of polymorphonuclear leukocytes (PMNs) and monocytes may play an important role in their phagocytic function. In addition, leukocyte adhesiveness may affect margination, diapedesis, and movement of cells within tissues. Therefore, it is surprising that relatively few models are available for examining this cell function (1–3). The present paper describes a simple, reproducible method for measuring leukocyte adhesion to glass capillary tubes. The method is sensitive, and the necessary equipment is available in most clinical laboratories.

Methods and Materials. Normal human blood obtained by venipuncture was anticoagulated with heparin in a final concentration of 3.3 mg/100 ml. Leukocyte-rich plasma (LRP) was prepared as follows. Samples of heparinized whole blood were centrifuged at 500*g* for 10 min, two-thirds of the lower red cell layer was removed with a pipet, and the remaining cells and plasma were mixed. After sedimentation of the remaining erythrocytes at 37° for approximately 1 hr, LRP was removed and centrifuged in 10 ml aliquots at 130*g* for 10 min. This sedimented the majority of the leukocytes. The platelet-rich supernatant was aspirated and replaced with the desired volume of autologous platelet-free plasma. The resulting cell suspension (LRP) was used to prepare test mixtures.

The method used is shown in Fig. 1: (a) Glass capillary tubes (10 per variable) were marked 5 mm from one end and completely filled with test mixture which had been incubated for 10 min at 37°. The marked end of each tube was sealed with a critocap. (b) Tubes were taped to large glass microscope slides. The superior surface of the open ends of tubes was marked to facilitate location of the inferior (cell-adherent) surface at a later time. (c, d, e) After incubation for 10 min in a horizontal position, tubes were centrifuged for 2 min at 11,500 rpm, scored and broken at the 5 mm mark. The smaller end with nonadherent cells was discarded. (f) Liquid content of the tubes was expelled and discarded. (g) Each capillary tube was immersed in 8 ml of isoton containing 10 m*M* EDTA. (h, i) After incubation for 15 min at 37°, each isoton-EDTA mixture with capillary tube was transferred to a 20 ml counting vial. An additional 12 ml of isoton were forced through the capillary tube into the vial using a 20 ml syringe and a snugly fitting, size 16, angiocath needle. (The solution must be forced through tubes in order to dislodge all adherent cells). (j, k) In order to lyse platelets, 6 drops of zap isoton were gently mixed with the contents of each vial shortly before white blood cell counts were made with a Model F Coulter counter (4). Background counts of the test mixtures were determined by centrifuging the test mixtures after cell counts had been made, using the cell-free supernatant to fill capillary tubes (2 per variable), and processing the capillary tubes as described above (mean background counts for Table I were 405 ± 39 SEM). The mean background count for a particular test mixture was subtracted from each cell count of that mixture. Counts greater than 10,000 were corrected according to the Coulter counter coincidence correction chart (4). The mean of 10 counts for each variable was designated as the adhesion index (AI) for that variable. In order to

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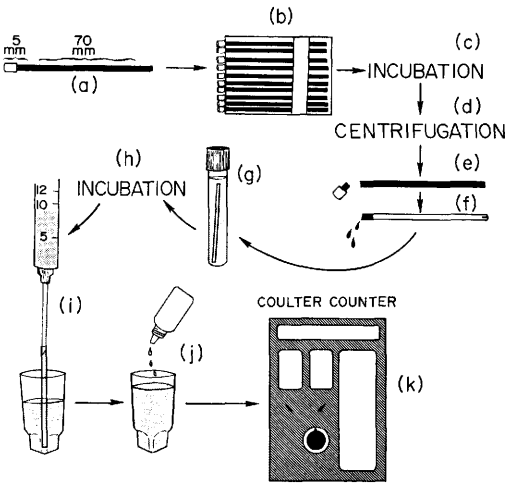

FIG. 1. Method for the study of leukocyte adhesion to glass; see text for details.

determine the total number of leukocytes in a particular test mixture, the mixture was loaded into a capillary tube to a premarked point, contents immediately expelled into the isoton-EDTA solution, and count determined as described above. The appropriate background count was subtracted from the machine count and the result corrected if greater than 10,000. The resulting value has been designated as the test white blood cell (WBC) count of that mixture.

Adhesion was expressed as percentage of control adhesion or as percentage of test WBC count:

$$\% \text{ of control adhesion} = \frac{\text{test AI}}{\text{control AI}} \times 100,$$

$$\% \text{ of test WBC count} = \frac{\text{test AI}}{\text{test WBC count}} \times 100.$$

Verification that virtually all formerly adherent cells had been rinsed from the capillary tubes into the counting vials was attained by microscopic examination of at least one rinsed tube per variable.

Differential counts. Differential counts of white cells adhering to capillary tubes were determined in the following manner: Tubes containing adherent cells were emptied of test mixture, filled with Wright's stain by capillarity, and subsequently refilled with a mixture of stain and buffer (phosphate buffer, pH 6.8). Tubes were rinsed with tap water, dried with a jet of air, filled with permount, taped to a microscope slide, and the differential count made microscopically at 1250×. In control preparations cells were exposed to Wright's stain for approximately 1 to 1.5 min and to stain–buffer mixture for 2 to 3 min. Cells from noncontrol test mixtures usually had different staining characteristics and times were adjusted accordingly.

Special equipment, reagents, and glassware. Preservative-free heparin (Connaught Laboratories, Westlake, Ontario) was prepared in sterile isotonic saline. EDTA, $NaAsO_2$ (Baker), EGTA, and MgEGTA (Geigy Industrial Chemicals) (5) were dissolved in sterile distilled water. The Coulter counter was obtained from Coulter Electronics (Hialeah, FL). Osmolalities were measured on an Advanced Instruments osmometer. Isoton and zap isoton were supplied by Coulter Diagnostics, Trizma base by Sigma Chemical Co., critocaps by Sherwood Medical Industries, capillary tubes (capilets) by Scientific Products, and Millipore field monitors by the Millipore Corp. (Bedford, MA). All glassware was acid cleaned and, with the exception of capilets, siliconized (Siliclad, Clay Adams). All glassware was sterilized in a dry oven at 190° for 2 hr.

Experiments. The effects of a number of factors on leukocyte adhesion to glass were determined in order to test the effectiveness of this method. Only modifications of the basic technique as described above are noted. Adhesion was expressed as percentage of control or percentage of test WBC count.

Temperature. Samples of LRP were incubated for 15 min at 16, 24, and 37°. Capillary tubes were filled and incubated for 60 min at the same temperature as the sample used to load them. Background count was made from the specimen incubated at 37° (control).

Divalent cation depletion of plasma. Test mixtures of LRP contained either 10 m*M* EGTA, 10 m*M* MgEGTA, or an equal volume of isotonic saline.

Hyperosmolality. Test mixtures were made hypertonic by addition of 1.2 *M* NaCl to LRP in a ratio of either 1:24 (osmolality =

362 mOsm/kg), or in a ratio of 1:6 (osmolality = 602 mOsm/kg). Isotonic saline was added to LRP in the control specimen in a ratio of 1:6. Volumes were equalized by addition of isotonic saline.

Sodium arsenite. Two test mixtures of LRP containing 0.1 m*M* $NaAsO_2$ were prepared. One was used immediately to fill a set of capillary tubes. The second was rotated for 1 hr at 37° before being loaded into capillary tubes. A control specimen containing isotonic saline was prepared for each sodium arsenite variable and treated similarly.

Heated plasma. In order to test the effect of heated plasma on leukocyte adhesion to glass two cell pellets were washed twice in heated plasma. One pellet was resuspended in heated plasma and the other in fresh plasma. The control cell pellet was washed twice and resuspended in fresh plasma. The heated plasma was prepared by heating sterile, autologous, heparinized plasma at 56° for 30 min, centrifuging at 1000*g* for 10 min, and discarding the sediment. White cell pellets were prepared by centrifuging 3 ml of LRP at 130*g* for 10 min and removing the supernatant. The cell washing procedure involved resuspension of the pellet in the desired medium, thorough mixing with a sterile pipet, centrifugation at 130*g* for 10 min, and discarding the supernatant. Cell pellets were washed twice before being resuspended in the final test mixture.

Adhesion in sucrose and in saline solutions. Four solutions were prepared: Sucrose solution 1 contained 0.27 *M* sucrose, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 5 m*M* Trizma base. Sucrose solution 2 contained 0.27 *M* sucrose and 5 m*M* Trizma base. Saline solution 1 contained 0.35 *M* NaCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 5 m*M* Trizma base. Saline solution 2 contained 0.135 *M* NaCl and 5 m*M* Trizma base. The ingredients for each solution were dissolved in glass distilled water and the pH was adjusted to 7.5 with 6 *N* HCl. All solutions were heparinized in a final concentration of 6.6 mg/100 ml and sterilized by filtering through a Millipore field monitor of 0.45 μ pore size. The solutions were used to wash and resuspend cell pellets. The washing procedure and cell pellet preparation have been described above. The control pellet was washed and resuspended in fresh, autologous, heparinized plasma. A nonwashed sample of LRP was also included.

Adhesion of untreated or control LRP had a range of 6 to 35% of the test WBC count. In general, the adhesion index increased as the test WBC was increased (6). As shown in Table II, white counts of these washed leukocyte mixtures differed considerably. Therefore, adhesion was expressed as percentage of test WBC counts.

Results. Preliminary studies indicated that heparin, in concentrations as high as 24 mg/100 ml, exerted no discernible effect on adhesion. It was also demonstrated that use of siliconized capillary tubes resulted in increased adhesion (6), but adherent cells were less susceptible to complete rinsing from siliconized tubes. On the other hand, nonsiliconized capillary tubes could be consistently rinsed free of adherent cells. In addition, siliconized tubes did not fill by capillarity. Therefore, we elected to use nonsiliconized capillary tubes.

Differential white blood cell counts of untreated LRP used in these studies showed approximately 60% neutrophils, 30% lymphocytes, 7% monocytes, and 3% eosinophils and basophils. Differential counts done on adherent cells from untreated LRP in capillary tubes showed a decrease in the lymphocyte count, often to zero, with a proportionate rise in the monocyte count. The percentage of neutrophils, eosinophils, and basophils remained essentially the same as that of the LRP used to fill the tubes.

Temperature. As shown in Table I, leukocyte adhesion was reduced at temperatures lower than 37°. Adhesion could not be tested at 4° because particulate matter formed in plasma when tubes were incubated at that temperature, resulting in false counts on the Coulter counter. When these specimens were examined microscopically, however, no cells were seen. Thus, cells do not appear to adhere to glass at 4°.

Divalent cation depleted plasma. As indicated in Table I, leukocyte adhesion to glass was abolished in the presence of 10 m*M*

EGTA. However, in 10 m*M* MgEGTA leukocyte adhesion was enhanced.

Hyperosmolality. Increasing plasma osmolality to 362 mOsm/kg with NaCl augmented leukocyte adhesion to capillary tubes (Table I). Contrarily, increasing osmolality to 602 mOsm/kg caused a marked inhibition of cell adhesiveness.

Sodium arsenite. Leukocyte adhesion was enhanced when cells were exposed briefly to 0.1 m*M* $NaAsO_2$. A longer period of exposure to the same agent, however, considerably impaired adhesion (Table I). Although not shown in Table I, adhesion was also abolished by exposure of cells to 10 m*M* sodium arsenite for 1 hr. This treatment caused cell death as indicated by membrane permeability to trypan blue.

Heated plasma. As shown in Table I, leukocyte adhesion was reduced when cells were washed and resuspended in heated plasma. Furthermore, even the process of washing cells in heated plasma seemed deleterious to leukocyte adhesion because resuspension in fresh plasma did not restore normal adhesive capacity to cells washed in heated plasma.

Adhesion in sucrose and saline solutions. Adhesion was increased by washing and resuspending leukocytes in each of the relatively plasma-free mixtures (Table II). The sucrose and saline test mixtures containing Ca^{2+} and Mg^{2+} produced a greater degree of adhesion than comparable solutions without those cations. Examination of capillary tubes prior to removal of adherent cells showed many large aggregates of cells in tubes containing sucrose solution 1. Sucrose solution 2 test mixtures usually became viscous with formation of strands of solid material.

Discussion. The present method has several advantages over previous techniques (1–3). In contrast to procedures employing glass bead columns, only adherent cells are measured and artificial elevation of adhesion due to sequestration of cells can be excluded. Furthermore, cells in capillary tubes can be examined directly with a microscope, and differential counts and characteristics such as cell aggregation can be noted. In addition, other characteristics of adherent cells can be examined after cells have been removed from capillary tubes. Moreover, it is possible to verify that all adherent leukocytes have been removed from capillary tubes by examining rinsed tubes microscopically. The present method is simple, materials are inexpensive, and equipment is readily available in clinical laboratories. Many observations can be made for each variable and a number of variables can be measured in a single test. Thus, statistical reliability is increased.

In order to confirm the adequacy of the present method, a number of factors known to affect leukocyte adhesion to glass were studied. Fenn (1), Garvin (2), and Kvarstein (7) noted that optimal adhesion occurs at 37° and that adhesiveness is reduced as temperature is lowered. Similar findings were observed in the present study (Table I). The effect of divalent cation depletion on leukocyte adhesion was confirmed with the present method. Adhesion was abolished in 10 m*M* EGTA but was enhanced in 10 m*M* MgEGTA (Table I). These results appear to reflect enhanced adhesiveness of leukocytes in the presence of increased magnesium ion concentration and imply that leukocyte adhesion can occur in the virtual absence of calcium ion (5, 8). Similar findings have been described with regard to platelet adhesiveness (9). Stimulation of leukocyte adhesion to glass is also noted in plasma made slightly hypertonic with sodium chloride. This finding may have relevance to leukocyte function in the environment of the renal medulla during water diuresis. Studies by Kvarstein suggested a similar relationship between osmolality and adhesion, but his studies were done in plasma-free media (10).

It is not known if dead leukocytes can adhere to glass. However, results obtained with 0.1 m*M* sodium arsenite suggest that during the early period of cell injury leukocyte adhesiveness may be enhanced. Prolonged injury, on the other hand, such as that incurred by rotation of cells for 1 hr in 0.1 m*M* sodium arsenite, leads to almost complete inhibition of adhesion, suggesting that nonviable cells cannot adhere to glass.

The effect of heated plasma on leukocyte adhesion to glass is controversial. Kvarstein (10) found that mean adhesion was higher in

TABLE I. Factors Affecting Leukocyte Adhesion to Glass.[a]

Factor being tested	Adhesion index[b]	SEM[c]	Percentage of control adhesion[d]
Temp (°)			
16	742	70	27
24	1680	104	62
37	2706	188	100
Divalent cation depleted plasma			
10 m*M* MgEGTA	6218	138	117
10 m*M* EGTA	0	0	0
Isotonic saline	5316	118	100
Hyperosmolality (mOsm/kg)			
362	4058	306	252
609	22	15	1
287	1610	165	100
Sodium arsenite			
0.1 m*M* sodium arsenite	7833	162	147
no rotation			
Isotonic saline	5330	210	100
0.1 m*M* sodium arsenite	368	110	8
1 hr rotation			
Isotonic saline	4448	116	100
Heated plasma			
Washed in heated plasma, resuspended in fresh plasma	1668	107	63
Washed in heated plasma, resuspended in heated plasma	7	6	0
Washed in fresh plasma, resuspended in fresh plasma	2638	61	100

[a] All values are means of 3 experiments.
[b] Mean of Coulter counts — background counts (corrected if >10,000).
[c] Standard error of the mean (SEM).
[d] (Test AI/control AI) × 100.

heat-inactivated serum. The present studies, like those of Penny and co-workers (12), demonstrated a marked impairment of adhesion in heat-inactivated plasma. As shown in Table I, even suspension of cells in heated plasma prior to adhesion in fresh plasma is associated with reduced adhesive capacity. The identity of heat-labile factors affecting leukocyte adhesion to glass is unknown.

Suppression of leukocyte adhesion in heated plasma and in plasma containing 10 m*M* EGTA suggests that this function is complement-dependent. Enhanced adhesion in the presence of 10 m*M* MgEGTA argues against the participation of the whole complement sequence (5) although not against the participation of later acting complement components as isolated proteins. The finding that leukocyte adhesion is enhanced in plasma-free media with or without divalent cation (Table II) suggests that one major role of plasma proteins may be prevention of leukocyte adhesion within the vascular system. Viewed in this light, changes in plasma proteins due to infection or other trauma might be considered as altering this normal antiadhesive effect of the normal plasma milieu.

Fidalgo and Najjar have suggested that washing leukocytes in saline containing media removes a gamma globulin fraction

TABLE II. Leukocyte Adhesion to Glass in Sucrose or Saline Solutions.[a]

Treatment of cells	Test WBC count[b]	Adhesion index[c] ± SEM[d]	Adhesion (%) of test WBC count[e]
Washed and resuspended in 0.27 *M* sucrose, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* Trizma base	8602	6968 ± 515	81
Washed and resuspended in 0.27 *M* sucrose, 5 m*M* Trizma base	5327	1580 ± 256	30
Washed and resuspended in 0.135 *M* saline, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 5 m*M* Trizma base	13,400	9807 ± 190	73
Washed and resuspended in 0.135 *M* saline and 5 m*M* Trizma base	13,900	7875 ± 715	57
Washed and resuspended in fresh plasma	15,100	1390 ± 88	9
Nonwashed plasma control	19,400	2175 ± 202	11

[a] All values are means of 3 experiments.
[b] Test WBC count — background count (corrected if >10,000).
[c] Mean of Coulter counts — background counts (corrected if >10,000).
[d] Standard error of mean (SEM).
[e] (Test AI/test WBC count) × 100.

necessary for optimal phagocytosis (13). Low ionic strength sucrose solutions did not have this effect (14). In the present studies adhesive capacity of cells washed either in saline or in sucrose solution was enhanced. This finding suggests that the gamma globulin postulated to be necessary for phagocytic function is not necessary for leukocyte adhesion to glass. On the contrary, previous studies have demonstrated that leukocytes can ingest opsonized bacteria in concentrations of EDTA which abolish leukocyte adhesion to glass (8). Thus, although adhesion may be an important component of the phagocytic process, it is probable that phagocytosis is influenced by a number of factors independent of those affecting cell adhesion. Further studies are needed to determine the role of leukocyte adhesion in the phagocytic process.

Leukocyte adhesion to glass may represent an *in vitro* counterpart of leukocyte adhesion to vascular epithelium or other tissues *in vivo*. Although this consideration may seem overly presumptive, there are several lines of evidence suggesting that the two phenomena may be similar in certain respects. In plasma leukocyte adhesion to glass is increased by the same experimental manipulations which enhance leukocyte adhesion to other leukocytes or to bacteria. These manipulations include rotation of cells with endotoxin, antigen–antibody complexes, or bacteria, (3, 14), slight increase in plasma osmolality, or a slight increase in magnesium ion concentration. Leukocyte adhesion to glass is decreased under conditions which inhibit leukocyte adhesion to other leukocytes or to bacteria. This is observed after divalent cation chelation by addition of EDTA to plasma (3), after inactivation of plasma by heat, and in the presence of sodium arsenite (3). It seems reasonable to suggest, therefore, that adhesion of leukocytes to glass involves at least some of the same factors affecting leukocyte aggregation and adhesion to particulate matter and tissues. Examination of those factors by the system presented herein may offer valuable insight into the physiology of leukocyte adhesion.

Summary. A new method for quantitation of leukocyte adhesion to glass is described. Using this method, it was possible to demonstrate the adverse effects of low temperature, sodium arsenite, divalent cation chelation, marked hyperosmolality, and heat inactivated plasma on leukocyte adhesiveness. Adhesion was enhanced by increased Mg^{2+} concentration, minimally increased concentrations of sodium chloride, brief exposure to sodium arsenite, and suspension of leukocytes

in a plasma-free environment.

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