Determination of the role of cuticular carbohydrates in the hemocompatibility of *Dirofilaria immitis* (Nematoda)

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We have partially characterized surface glycoproteins of the canine heartworm, *Dirofilaria immitis*. Histochemical studies indicated the presence of neutral and acidic mucopolysaccharides at the blood–cuticle interface. Fluorescein isothiocyanate-conjugated lectin binding patterns suggested the presence of α-D-glucosyl and/or α-D-mannosyl, β-galactosyl, *N*-acetylneuraminyl and *N*-acetylated-D-hexosaminyl (sialic and glucuronic acids, respectively) terminal residues among the constituent sugars of the glycocalyx. An additional goal of this study was to assess the significance of each carbohydrate in parasite hemocompatibility by using scanning electron microscopy, internal reflection infrared spectroscopy, and comprehensive contact angle measurements. Each carbohydrate identified in the glycocalyx was selectively cleaved with the appropriate exoglycosidase. Heartworms bearing native and enzyme-altered surfaces were exposed to platelet-rich canine plasma. Activation and aggregation of platelets were significantly increased on enzyme-treated surfaces as compared with native surfaces. Enzyme-induced cleavage of carbohydrate residues was associated with an increase in critical surface tension or a loss in cuticular structural integrity or both. Hemocompatibility of the heartworm cuticle depends on the retention of a stable saccharide-rich layer that minimizes interaction with plasma proteins and platelets; thus, carbohydrate residues on the glycocalyx may contribute to parasite hemocompatibility. The presence of similar low-critical-surface-tension coatings with high mechanical integrity may impart thromboresistance to other polyphenolic or chitinous substances. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

Dirofilaria immitis is an important pathogen for dogs; and in many parts of the world, it is pathogenic for other mammals, including humans.[1,2] Canine filariasis is a good model for the study of vascular response to chronic injury[3,4] because intravascular infection results in a typical pattern of endothelial cell damage and loss followed by platelet–leukocyte adhesion and myointimal proliferation. Clinical and laboratory investigations have suggested that the acellular nature of the *D. immitis* cuticle and its thromboresistant surface properties allow the parasite to circumvent the host's immune response and improve long-term survival. However, the characteristic nonthrombogenicity of the heartworm cuticle has not been thoroughly investigated.[5,6] The study of this unique host–parasite relationship may yield an understanding of the principles underlying the remarkable inertness of the parasite. Furthermore, this information may be useful in developing more hemocompatible blood-contacting biomaterial surfaces.

The basic morphology, biochemistry, and physicochemistry of the *D. immitis* cuticle have been investigated by Bilge et al.[7] They have demonstrated that the proteinaceous coat at the blood–cuticle interface is composed of host proteins that can easily be stripped off the cuticle in acidic or ionic medium. These findings were suggestive of weak ionic interactions rather than strong covalent bonds at the interface. In addition, Bilge et al. have provided evidence for a glycocalyx-like outermost layer. However, there has been no attempt to characterize the glycoprotein content of the glycocalyx or to define the role of its constituent sugars in parasite hemocompatibility.

Mono- or polysaccharides on nematode surfaces may play a role in important biological functions. Complex sugars may contribute to para-

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site nonthrombogenicity.[8-10] Simple monosaccharides, such as mannose and fucose, and glycoprotein moieties, such as sialic and hyaluronic acids, are involved in regulatory behavioral interactions, including nematode chemotaxis[11,12] and entrapment by nematophagous fungi.[13,14] These cuticular components also prevent desiccation of free-living nematodes[15] and provide binding sites for concentrating electrolytes from the environment.[16]

Such parasite factor(s) may allow the heartworm to evade entrapment in blood clots by interfering at one or more stages in the intrinsic and extrinsic coagulation sequence of the host. The host protein layer at the blood–parasite interface and terminal carbohydrate residues of the adult worm's glycocalyx may contribute to parasite survival.[7,17] This study was therefore conducted to partially characterize the sugar content of the *D. immitis* cuticle and to identify the significance of each carbohydrate species in cuticular hemocompatibility.

MATERIALS AND METHODS

Histochemical Analysis

Fresh worms were rinsed in culture medium RPMI-1640 (Gibco Ltd., Grand Island, NY) and kept frozen at −20°C. Thawed worms were fixed in 10% formaldehyde for 3h, dehydrated, processed in paraffin, and stained with PAS-Alcian blue for polysaccharides and mucosubstances.[18] A dog footpad was used as control. Sections were viewed under a Zeiss Optical Microscope.

Carbohydrate Analysis

Fluorescein isothiocyanate (FITC)-conjugated lectins (Sigma Chemical Company, St. Louis, MO) were used to label the glycocalycal carbohydrates. Lectins and the experimental conditions in which they were used were chosen as follows: Concanavalin A (Con A), which has an affinity for alpha-D-mannosyl and alpha-D-glucosyl residues, was used in 0.01*M* phosphate-buffered saline (PBS, pH = 6.8) containing 0.01 m*M* $CaCl_2$ and 0.01 m*M* $MnCl_2$. *Ricinus communis* agglutinin (RcA), specific for terminal β-galactosyl residues, was incubated in 0.01*M* PBS, pH = 6.8. *Ulex europaeus* agglutinin (UeA), the first type (UeA-I) having an affinity for L-fucose, was reacted in 0.01*M* PBS, pH = 6.8. *Limulus polyphemus* agglutinin (LpA), which has an affinity for *N*-acetylated-D-hexosamines and for sialic acid, was used in 0.05*M* Tris buffer, pH = 8.0, containing 0.01*M* $CaCl_2$.

A 50-μg/mL solution of each lectin–FITC conjugate was prepared in the appropriate buffer. Frozen male worms were prepared for microsectioning. Sections of 5-μm thickness were placed on glass slides, deparaffinized, and rinsed in distilled water for 20 min. Each section was incubated in one drop of the lectin–FITC conjugate in buffer in the dark for 1 h at room temperature. Following three 15-min rinses, sections were examined under a Zeiss fluorescence microscope equipped with an ultraviolet filter.

Positive and negative controls were used for each lectin–FITC conjugate. For positive controls, fresh human red blood cells (RBCs) were incubated in each lectin–FITC conjugate and were observed for agglutination and fluorescence. RBCs were prepared by drawing 1 mL of blood into a vacutainer tube (Becton Dickinson Vacutainer Systems, Rutherford, NJ).[18] Normal buffered saline was added, and the cells were centrifuged. The plasma and buffy coat were discarded. Then, RBCs were washed three times and were suspended in PBS to a concentration of 6.7×10^8 cells/mL buffer (hematocrit of 3%). RBCs were incubated with lectin–FITC conjugate (50 μg/mL) in the dark for 1 h, rinsed, and washed three times. Cells were resuspended to a hematocrit of 3%, and blood smears were viewed under the fluorescence microscope. The procedure was repeated for each lectin–FITC conjugate.

A first set of negative controls was prepared by incubating each lectin–FITC conjugate in a 0.2*M* solution of the competitive sugar. The worm sections were then labeled with the inactivated lectins as described above. A second set of negative controls was prepared by selective enzymatic cleavage of single terminal sugar residues of the parasite glycocalyx. Worms were ligaclipped at both ends and incubated for 1 h with one of the following: 20 units/mL β-galactosidase in 10 m*M* PBS, pH = 7.45, 37°C; 20 units/mL α-glucosidase in 10 m*M* Tris buffer, pH = 7.2, 30°C; 50 units/mL L-fucose dehydrogenase in 10 m*M* Tris buffer, pH = 8.0, 30°C; 40 units /mL neuraminidase in 10 m*M* sodium acetate buffer containing 4 m*M* $CaCl_2$, pH = 5.5, 30°C; or 15,000 units/mL β-glucuronidase in 10 m*M* sodium citrate buffer, pH = 4.5, 30°C.

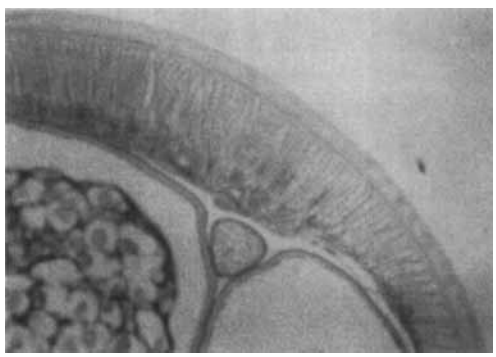


Figure 1. Light micrograph of a cross section of a heartworm stained with PAS-Alcian blue (original magnification ×40).

Experimental conditions and initial values for enzyme concentrations were adopted from Jansson et al.[14] The optimal concentrations for the actual system were determined by experimentation, by using a series of increasing enzyme concentrations and choosing the lowest value for which subsequent binding of the lectin was completely inhibited. Enzyme-treated worms were rinsed in the corresponding buffer twice for 15 min each, and worm segments approximately 1 cm long were transferred to 0.01*M* PBS (pH = 6.8). They were then sectioned and incubated in lectin–FITC conjugates as described above.

Surface Hemocompatibility Analysis

Each sugar identified as a constituent of the glycocalyx by the FITC labels was further investigated as to its role in the general hemocompatibility of the worm. The sugar was enzymatically cleaved, and the stripped worm was subjected to a platelet retention test.

Platelet Preparation

Platelet-rich plasma (PRP) was prepared according to the method described by Haslam and Rosson.[20] Briefly, dogs were administered Ace promazine 0.1 cm^3/kg body weight), and 40 mL of blood was drawn from the jugular vein into 10 cm^3 polystyrene centrifuge tubes containing a 3.8% (wt/vol H_2O) solution of sodium citrate tribasic for a final ratio of 9:1 (volume blood/volume anticoagulant). After centrifugation at 200*g* for 15 min, the platelet-rich supernatant was collected from each tube with polystyrene pipettes, pooled, and incubated at 37°C for 10 min. The plasma was used immediately.

After incubation with the enzyme, each worm was rinsed in the buffer corresponding to the enzyme with which it was treated. Worms were rinsed twice with buffers prewarmed to 37°C. Each enzyme-treated and rinsed worm was then placed in a well of a flat-bottomed polystyrene tissue culture plate (Multiwell, Becton-Dickinson, NJ) where it was incubated in prewarmed PRP at 37°C. Individual incubation times varied: incubation periods of 15 s, 1 min, 3 min, 10 min, 30 min, and 1 h were used. Worms were then directly transferred into 2.5% glutaraldehyde in 0.01*M* PBS, pH = 7.4, prewarmed to 37°C, where they were allowed to cool to room temperature for 3 h. After a change with cold fixative, worms were kept in the glutaraldehyde solution for 36 h at 4°C and processed for scanning electron microscopy. Throughout the experiment, an untreated worm (i.e., a dead worm with intact glycocalyx) and a precleaned glass microscope slide (Fisher Scientific, Pittsburgh, PA) served as negative and positive controls, respectively.

Scanning Electron Microscopy

Worms for scanning electron microscopic examination for platelet retention were passed through a series of alcohols of increasing concentrations and then soaked in absolute alcohol for 1 h, with two changes. Dehydrated worms were dried to the critical point using CO_2 as the transitional fluid.[21] Then, they were attached to stubs using double-sided tape, and the worms were coated with a 90-nm-thick layer of gold in a Hummer X sputter coater (Anatech Ltd, Battle Creek, MI). Coated specimens were stored in a desiccator until they could be examined with a JEOL JSM-IC848 scanning electron microscope at an accelerating voltage of 20 kV.

Contact Angle Measurements

Comprehensive contact angle measurements (rather than contact angle measurements with water alone) were made by applying single, small microdroplets of pure, diagnostic liquids to air-dried worm surfaces. The liquids were applied to the worm surfaces by using a fine, freshly flamed platinum wire. Worm surfaces were kept taut by suspending them over the empty central zone of a miniature embroidery-ring-like device in which the worm ends were clamped (the device was made by nesting two Teflon O rings). The following liquids were used: distilled water at surface tension = 72.4 dyn/cm at 20°C; glycerol, 64.8 dyn/cm; formamide, 58.9 dyn/cm; thiodiglycol, 53.5 dyn/cm; methylene iodide, 49.0 dyn/cm; 1-bromonaphthalene, 45 dyn/cm; α-methyl naphthalene, 39.3 dyn/cm; and dicyclohexyl, 32.7 dyn/cm.

The data were plotted as cosines of the measured average contact angle versus the surface tension. Data are presented in the linear format (Zisman plot), which allows extrapolation of the data to the point where the contact angle would just reach 0°. The surface tension intercept at this point, termed the "critical surface tension," relates to the specific surface composition of materials. The limited quantity of the available contact angle data reflects both the small surface area of the worms and the variations in microroughnesses; therefore, no further calculations were documented to suggest various other surface-energy-related terms from the contact angle data.

Infrared Spectroscopy

Infrared absorption spectra specific to the outermost 2000 Å of each type of heartworm surface (control, acid- and salt-treated, and variously enzyme-treated) were acquired by the multiple attenuated internal reflection infrared (MAIR) technique by using miniature germanium (mini-Ge) prisms (5 mm × 12 mm × 1 mm thick) in a special beam-condensing attachment

to a Perkin-Elmer Spectrometer (Model 1420). Distilled water was used to rinse worms free of storage solutions. While still wet, the worms (in groups of three or four) were gently clamped against the mini-Ge prisms. Sequential spectra were taken during the drying process while also compressing the specimens more tightly against the analytical prism face. The resulting spectra were of good quality, revealing infrared absorption bands characteristic of the organic components at each type of prepared worm surface.

RESULTS

Upon treatment with low-pH Alcian blue stain followed by oxidation with periodic acid and staining with periodic acid-Schiff (PAS) reagent, the outermost layers of the cuticle stained positive for polysaccharides and acidic and neutral mucosubstances. The basal layer showed aniline blue-positive striations (Fig. 1).

All lectins caused agglutination and fluorescence of human RBCs, indicating that treatment with FITC did not alter chemical properties of the lectins. All adult heartworm sections incubated with FITC-conjugated lectins showed positive staining with the exception of those treated with the UeA-conjugate. In the fluorescent-positive sections, the characteristic staining pattern was as follows. The structures contained within the pseudocoel fluoresced brightly as did the muscle layer and the ovaries (Fig. 2). The fluorescence in the hypodermis was slightly less than that observed in the internal organs, and it was almost completely absent in the basal and median layers of the cuticle. The epicuticle, however, exhibited a continuous circumferential staining which, at higher magnification, was associated with the 1-μm-thick outermost layer (Fig. 3). Inactivation of the lectin using the monomeric form of the competitive sugar, or preincubation of the whole heartworm body with the corresponding glycosidase (prior to sectioning and exposure to the fluorescent lectin), effectively removed the epicuticular fluorescence (Fig. 4). Because enzymes could not reach deeper tissues of ligaclipped heartworms, sugar residues located internal to the cuticle remained intact. Upon subsequent incubation with the conjugated lectin, these internal residues were exposed after sectioning and fluoresced.

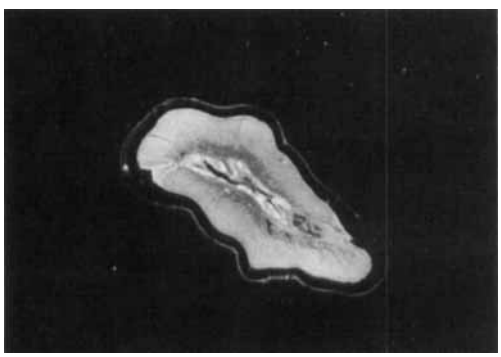


Figure 2. Fluorescence micrograph of a section of a whole heartworm treated with concanavalin A (original magnification ×25).



Figure 3. Fluorescence micrograph of a section of a whole heartworm treated with concanavalin A (original magnification ×100). Note the densely staining external cuticular layers (arrow).

In the case of the polyspecific lectins (Con A and LpA), controls were run for a combination of competitive sugars or glycolytic enzymes. Glucosidase treatment of the whole worm resulted in partial blockage of the binding of Con A, whereas incubation of fluorescein-conjugated Con A with 0.2*M* concentrations of monomeric mannose and glucose resulted in complete blockage. Similarly, preincubation of whole worms with glucuronidase or neuraminidase reduced the fluorescence, whereas treatment with glucuronidase followed by neuraminidase resulted in complete removal of staining from glycocalycal sites. Inactivation of the LpA in its complementary sugars (sialic and glucuronic acids) gave similar results. In RcA-treated heartworms, no fluorescence was observed when the lectin was inactivated by treatment with monomeric galactose or when the

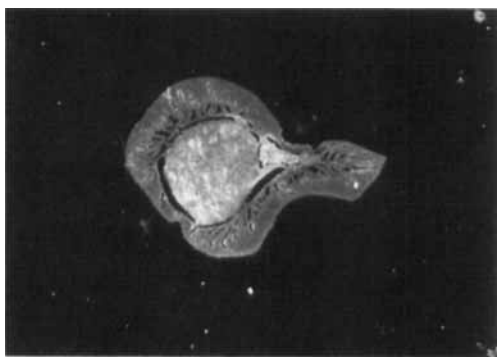
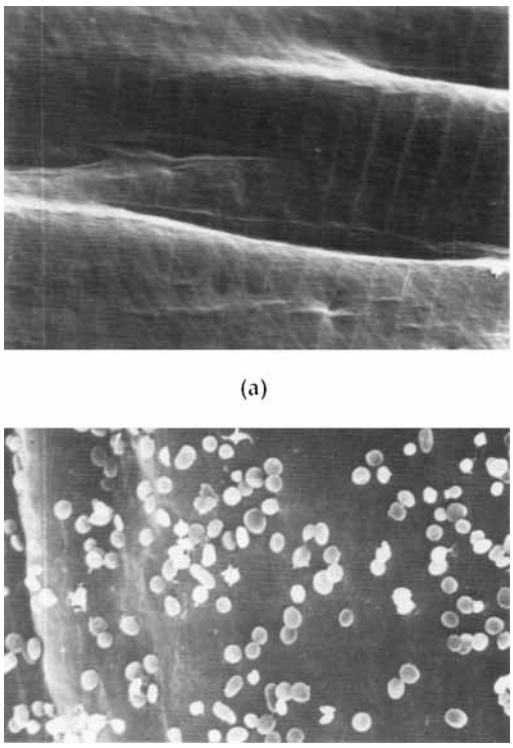


Figure 4. Fluorescence micrograph of a section of a heartworm treated with concanavalin A that had previously been inactivated with monomeric glucose and mannose (original magnification ×25).

glycocalycal galactose was removed by treatment with galactosidase. Sections stained with UeA, on the other hand, gave consistently negative results at the level of the glycocalyx.

Enzyme-treated heartworm surfaces were examined under the scanning electron microscope (SEM) after exposure to PRP. Enzyme-treated surfaces were compared with untreated, unexposed heartworm surfaces [Fig. 5(A)] and with untreated, exposed surfaces [Fig. 5(B)]. The degree of platelet transformation was qualitatively assessed by observing shape change, flattening, pseudopodal extension, and thrombocytolysis. Within 15 s of incubation, platelets started to adhere to all heartworm surfaces. Platelets that adhered to altered substrata immediately started to transform (Fig. 6). At the end of 1 h, aggregates composed of transformed platelets with pseudopods were observed on enzyme-treated surfaces (Fig. 7). The patterns of transformation were similar on neuraminidase-, glucuronidase-, and galactosidase-treated surfaces. In contrast, aggregates that were observed on native surfaces at the end of 1 h were composed of untransformed platelets with round, smooth surfaces (Fig. 8). These results indicated that platelet transformation depended on the nature of the surface involved.



(a) (b)

Figure 5. (A) Scanning electron micrograph of untreated, unexposed heartworm surface. (B) Scanning electron micrograph of platelets depositing on native heartworm surfaces after a 1-h incubation period (original magnification ×1500).

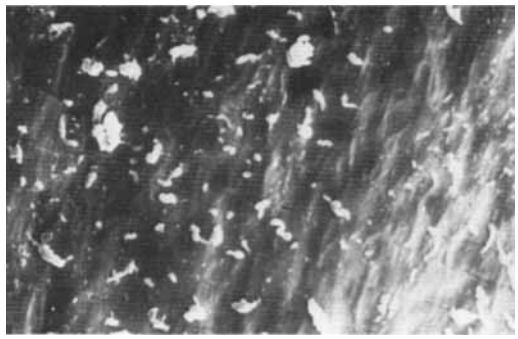


Figure 6. Scanning electron micrograph of platelets depositing on galactosidase-treated heartworm surfaces after a 15-s incubation period (original magnification ×1500).

Contact-angle data collected for the control heartworm cuticles that had not been treated with enzyme, salt, or acid were nonequilibrium values due to surface roughness caused by drying. Extrapolation of the limited number of data points (obtained by using high surface tension liquid—distilled water, glycerol, and thiodiglycol) yielded an estimated critical surface tension between 25–30 dyn/cm (Fig. 9), which is in agreement with the previously reported value of 27.7 dyn/cm.[7] The internal reflection infrared spectra of such surfaces also revealed the same glycoproteinaceous composition described earlier[7] as documented by absorption bands at 3400 and 3300 cm^{-1} (—OH and—NH), 2950 and 2850 cm^{-1} ($—CH_3$ and$—CH_2—$), 1640 and 1540 cm^{-1} (amide I and amide II bands, respectively), 1240–1260 cm^{-1} (hydroxylated rings, as in hydroxyproline or DOPA), and 1000–1100 cm^{-1} (—C—OH groups of polysaccharides). Very little residue was left on the analytical prisms after removal of control heartworm specimens, indicating a considerable degree of integrity of the glycocalycal sheath.

Salt-washing of heartworm cuticles significantly increased the critical surface tension, into the 30–40 dyn/cm zone, which is associated with thrombogenicity [Fig. 10(A)]. The MAIR-IR spectrum for the salt-washed heartworm cuticles illustrated that a matrix or



Figure 7. Scanning electron micrograph of platelets depositing on sialidase-treated heartworm surfaces after a 1-h incubation period (original magnification ×1500).

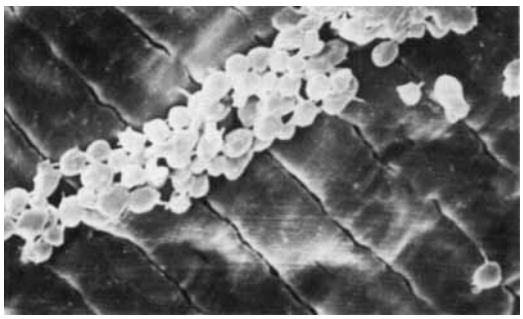


Figure 8. Scanning electron micrograph of platelet aggregates on native heartworm surfaces after a 1-h incubation period (original magnification ×950).

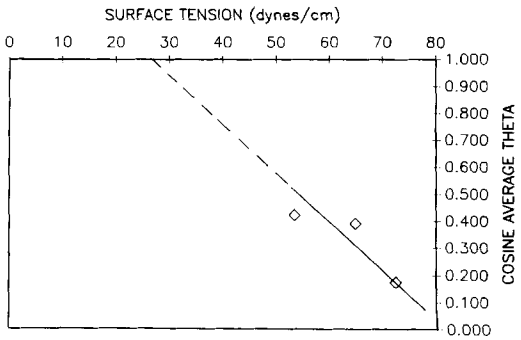


Figure 9. Zisman plot for control heartworm surface data.

filling substance had been removed to reveal the cuticular phenolic constituents [Fig. 10(B)]. Note specifically, the small sharp band at approximately 1240 cm^{-1} and the enhanced C-O_4 absorption region contours at approximately 1050 cm^{-1}. When salt-washed worm specimens were removed from the analytical prisms, they left behind a moderate residue of glycoproteinaceous fragments, illustrating the diminished structural integrity of the cuticle.

Contact-angle data for acid-washed heartworm cuticles demonstrated a substantial increase in critical surface tension. Note the steep slope of this plot, as also seen for the salt-washed specimens, and note that the gamma *c* intercept is about 40 dyn/cm. The MAIR-IR spectrum of the residue left behind on the analytical prism after removing the dried acid-washed specimens demonstrated the effective transfer of a substantial fraction of the acid-washed cuticular material to the contacting solid.

The combined process of salt-washing and acid-washing left the heartworm cuticles with critical surface tensions between 40–50 dyn/cm. Phenolic cuticular components were exposed, as indicated by the appearance of infrared bands at approximately 1240 cm^{-1} and 1050 cm^{-1}. However, this combined treatment resulted in a surprisingly lower transferability of cuticular substances to the analytical prisms.

Contact-angle data from the neuraminidase-treated heartworm cuticles were similar to those obtained with control specimens. Cuticles exposed to neuraminidase retained a low critical surface tension (between 20 and 30 dyn/cm) [Fig. 11(A)]. Nevertheless, the MAIR-IR spectrum for the neuraminidase-treated heartworms [Fig. 11(B)] showed a significant (spectral) exposure of previously masked phenolic sites, similar to that seen with salt-washed specimens. In contrast to the salt-washed specimens, however, the neuraminidase-treated heartworms transferred considerably less cuticular residue to the contacting analytical prism.

A contact-angle data plot for glucosidase-treated heartworms demonstrated an elevated gamma *c* (compared with controls) and an anomalously low water contact angle (compared with all other heartworm preparations examined). The MAIR-IR spectra for the glucosidase-treated group of heartworms revealed less unmasking of hydroxylated ring structures and very little residue on the analytical plates.

Contact angle results for galactosidase-treated heartworms were similar to those for glucosidase-treated specimens, but treatment with galactosidase resulted in considerably higher water contact angles. An enhanced C-OH absorption band was noticed in galactosidase-treated worms, and the specimen transferred a substantial cuticular residue to the test plates.

Contact-angle data for glucuronidase-treated heartworms [Fig. 12(A)] revealed increased critical surface tension that correlates with the uniformly increased infrared absorption (without an obvious plate) in the spectral regions from 1150 cm^{-1} to 1000 cm^{-1} [Figs. 12(B) and 13]. No residue was transferred from these specimens, indicating maintenance of cuticular structural integrity in the glucuronidase-treated heartworm cuticles.

DISCUSSION

In the present study, we have detected a carbohydrate-rich layer external to the adult *D. immitis* epicuticle by use of histochemical techniques. Our results with the PAS reagent are in accordance with earlier studies of cuticular carbohydrates stained with the ultrastructural dye, ruthenium red.[7] Previous reports have shown that this external layer is associated with a glycocalyx. We have used FITC-conjugated mono and polyspecific lectins to characterize partially the carbohydrate content of the cuticle. The specificity of the carbohydrate–lectin reaction was confirmed by inactivating each lectin with its competitive monomeric saccharide. When polyspecific lectins (Con A and LpA) were used, further resolution of the lectin-binding sites was achieved through selective removal of the involved species with glycolytic en-

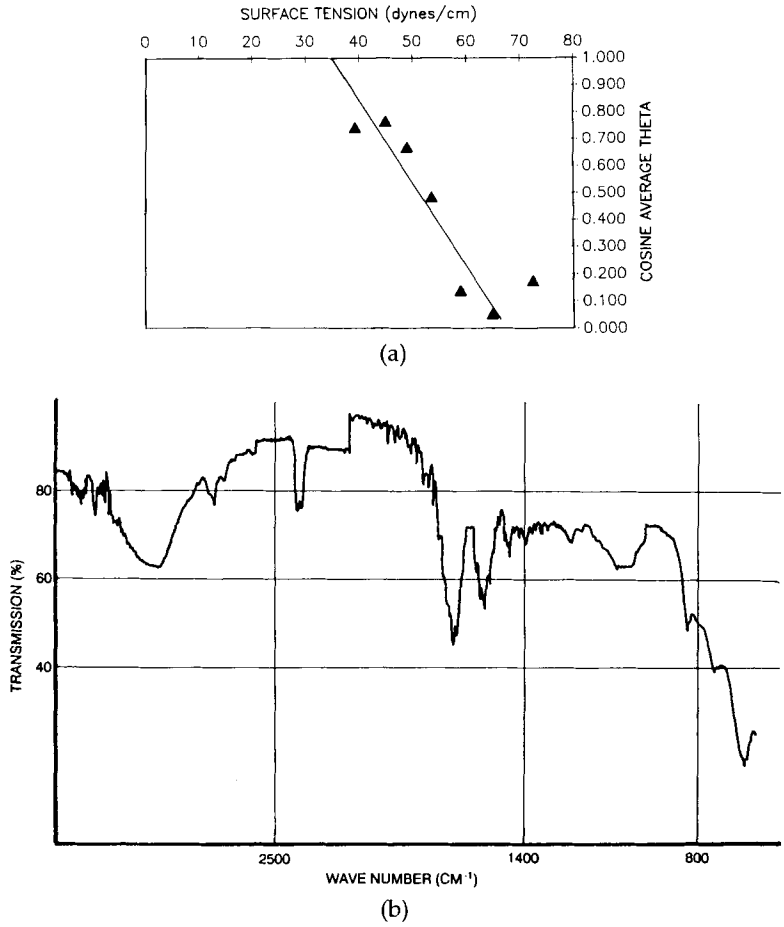


Figure 10. Salt-washed heartworm surface data: (A) Zisman plot, (B) MAIR spectrum.

zymes. Our results confirm the presence of galactose, glucose, mannose, sialic acid, and glucuronic acid.

The monosaccharides associated with the glycocalyx were enzymatically removed in order to assess their role in rendering the surface more hemocompatible. These sugar species probably contribute to the terminal sites of the plasmalemmar glycoproteins when an exoglycosidase is used. Such a procedure, therefore, is not done to obliterate the carbohydrate-rich matrix altogether, but it is an attempt to alter the identity or nature of the immediate blood interface of the parasite. This alteration may be more pronounced with the use of an endoglycosidase that will attack residues located further down the polysaccharide chain; therefore, the entire portion of the chain upstream is removed. But, in both cases, the treatment will uncover new carbohydrate species, different from the ones for which the enzymes are specific. Consequently, a clear advantage of the technique used here is that it provides an efficient means for removing any sugar residue from the interfacial zone so that the role of that specific residue in a given surface-related mechanism can be assessed.

In the present study, we used exoglycosidases to remove the terminal carbohydrate residues at the nematode surface. Then, the transformed surfaces were subjected to a modified platelet-retention test. Effects of these transformations were significantly more pronounced in platelets that had adhered to enzyme-treated surfaces than in platelets that contacted only native heartworm surfaces. This difference was detected as early as 15 s after first exposure, and, at 1 h, platelet lysis and shape change on altered cuticular surfaces exceeded those on glass surfaces. Such a strong reaction might be expected after the removal of the charged residues, namely, the neuraminic and glucuronic acids, that could exert an electrostatic repulsive force upon the negative surface charge of the platelet membrane. Indeed, earlier experiments with various nematode species have shown that destruction of the surface charge with neuraminidase,[22] hyaluronidase, or glucuronidase[23] facilitates the attachment of leukocytes to cuticular surfaces and the destruction of the cuticle *in vitro*.[24] The cleavage of glucose and galactose, however, had similar and unexpected platelet activation effects. Since ionic repulsion by these neutral residues is unlikely, polar–polar interactions may have played the main role in initiating platelet activation. Both glucosidase and galactosidase increased the critical surface tensions of the treated cuticular surfaces, in the same

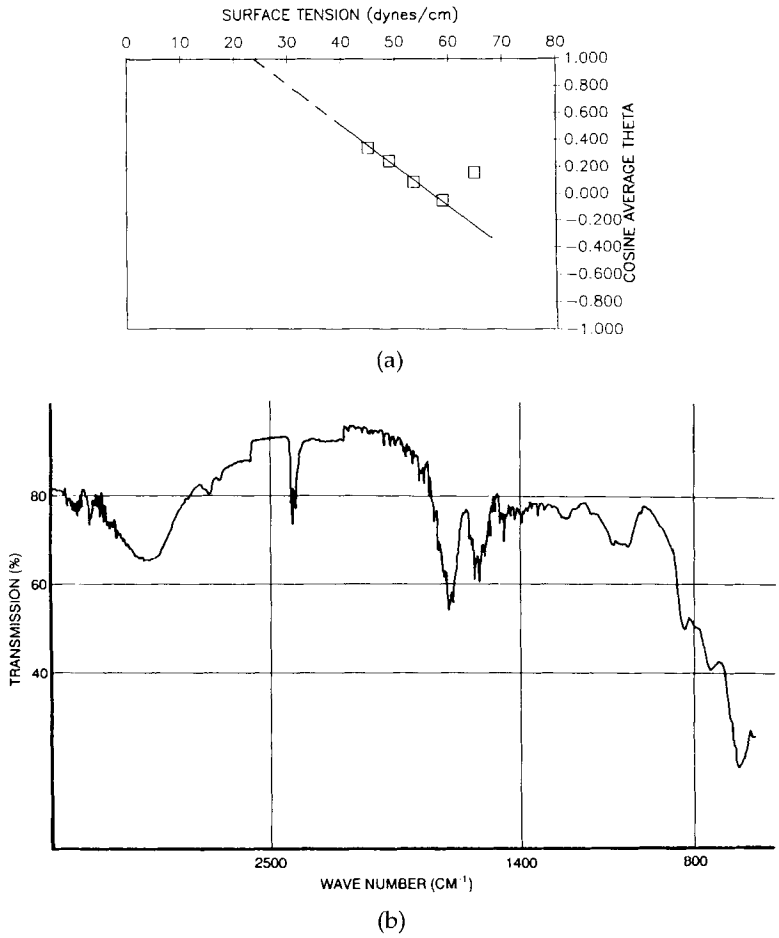


Figure 11. Neuraminidase-treated heartworm surface data: (A) Zisman plot, (B) MAIR spectrum.

direction, but not to the same degree as did salt-washing and acid-washing. This observation suggests that the platelet activation results are consistent with previous observations for these higher-energy surfaces.

It has been suggested that during prolonged static exposure of relatively inert biosurfaces, platelet aggregation is free to proceed in the suspension medium (even though the platelet concentration is not necessarily depleted through activation and surface adsorption) until a critical mass is reached, beyond which aggregates are sedimented toward the surfaces.[25] For the long-term exposures used in our study, this phenomenon may have interfered with the ability of quantitative cell counting and radiolabeling methods to assess the hemocompatibility of the biosurface. In addition, variations in the diameter and length of heartworm segments and the random cuticular microtopographical patterns (variations in parasite surface area) may have affected the generation of quantitative data. Therefore, we restricted our evaluation of platelet–heartworm interactions to qualitative tests only.

MacCormick[17] investigated the adult cuticle and found evidence of a surface protein adsorbate that significantly increased the hemocompatibility of the nematode *in vitro* as indicated by a more pronounced platelet response to ionically destabilized worm surfaces. Acid or salt extraction of the host proteins was later shown to alter the surface energy of the parasite.[7] Our results suggest a similar role for the glycocalycal carbohydrates of the cuticle. Enzymatic treatment of the parasites led to significant alterations of the critical surface tensions when the terminal glucuronic acid residues, the glucosidyl residues, and the galactosidal residues were removed. The similarity in platelet reactivity on neuraminidase-treated specimens, which did not significantly increase critical surface tension, was probably associated with the effective exposure of cuticular phenolic groups [compare Figs. 11(B) and 13] and weakening of the superficial layer as noted in its ease of component transfer to the analytical prisms.

Contact-angle data collected for the control heartworm cuticles, never exposed to enzyme, salt, or acid treatment, were similar to those presented by Bilge et al.[7] Removal of the control heartworm specimens from their compressive contacts with the analytical prisms left very little residue on the prisms, indicating a considerable degree of integrity of the initial glycocalycal sheath. Thus, combined histological, biochemical,

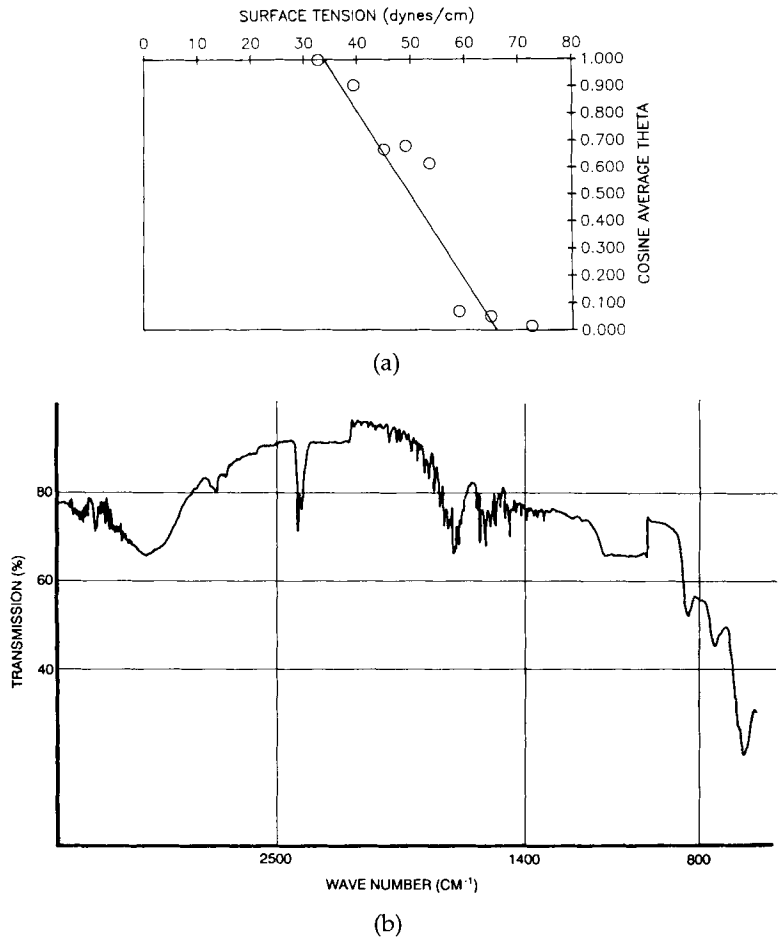


Figure 12. Glucuronidase-treated heartworm surface data: (A) Zisman plot, (B) MAIR spectrum.

immunostaining, and physicochemical data have established the following: (a) exposed heartworm cuticular carbohydrates provide a hemocompatible surface, as assessed by platelet reactivity assays; (b) destruction of these sites by salt-washing, acid-washing, or enzyme-cleavage of terminal residues is associated with both increased critical surface tensions and increased platelet reactivity.

These results also indicate that the physical and mechanical integrity of the cuticular surface is important in the maintenance of hemocompatibility. Thus, natural heartworm surfaces seem to maintain their

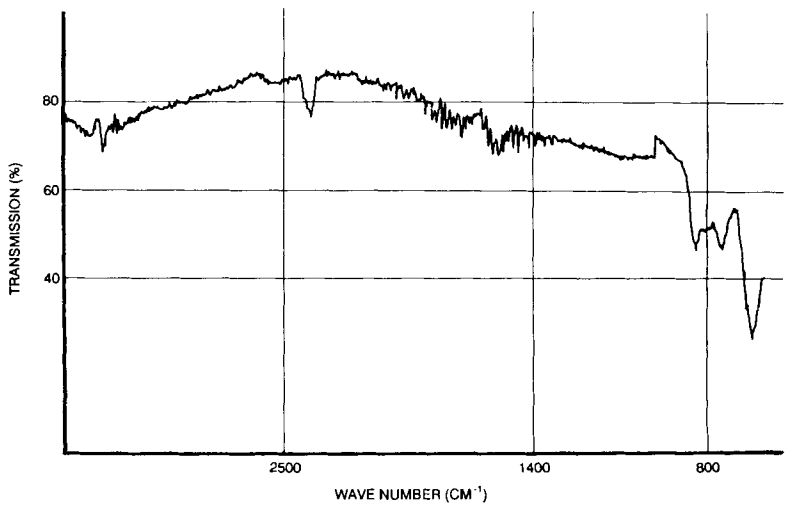


Figure 13. Prism-baseline MAIR-IR data.

long-term thromboresistance by exhibiting a stable, low-critical-surface-tension array of cuticular saccharide end-groups that react only benignly with adsorbing plasma proteins and subsequently arriving platelets.

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