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Identification of capillaries in sections from skeletal muscle by use of lectins and monoclonal antibodies reacting with histo-blood group ABH antigens

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This study was performed to evaluate the application of different lectins and monoclonal antibodies against ABH antigens to detect and characterize carbohydrate structures in capillaries of skeletal muscle from humans and laboratory animals. Blood group specific lectins (*Griffonia simplicifolia, Griffonia simplicifolia* isolectin B4, *Lotus tetragonolobus, Ulex europaeus*, and *Dolichos biflorus*) and monoclonal antibodies reacting with histo-blood group carbohydrate antigens belonging to type 1 (Le^a) and type 2 (H, A and Le^y) chains were used as histological markers for capillaries in sections from skeletal muscle. The material consisted of 20 human masseter muscle biopsies from individuals with known blood types: (eight blood group O, nine blood group A, two blood group B, and one blood group AB) and masseter muscles specimens from different laboratory animals (mouse, rat, rabbit, cat, dog, pig, cow, and macaca monkey). Unfixed sections and an avidin alkaline phosphatase method were used to visualize the specific reaction. *Ulex* lectin stained capillaries in all human biopsies either strongly or moderately. Strong muscle capillary reaction was observed in biopsies from O, B and AB individuals while capillaries from A individuals were only moderately stained. *Griffonia simplicifolia* marked capillaries in A, B, and AB individuals and *Griffonia simplicifolia* isolectin B4 stained capillaries in muscle biopsies from B and AB donors. *Dolichos biflorus* was a weak marker of muscle capillaries from A individuals. Only capillaries from O individuals were stained with the antibody against H type 2. Capillary reaction was not observed with the other antibodies used. *Girffonia simplicifolia* was an excellent marker for capillaries in mouse muscle while *Griffonia simplicifolia* isolectin B4 is recommended for rat muscles. Periodic acid treatment and subsequent *Lotus tetragonolobus* staining is suitable to visualize capillaries in mouse, rat and pig muscle. Using a sensitive histochemical technique for staining with lectins and monoclonal antibodies reacting with blood group related antigens the microvascular density in human skeletal muscle may be estimated. Further, the carbohydrate compounds in the muscle capillaries reflect the individual blood type. A selection of lectins is suitable for demonstration of capillaries in animal skeletal muscle.

Keywords: muscle capillaries, blood group carbohydrate antigens, lectins, monoclonal antibodies, histochemistry

The glycosylation of endothelial cells is highly specific [1, 2] and the glycoconjugates on endothelial cells (BAEC) might be involved in a number of functions such as selective uptake of proteins from the blood plasma to the interstitial fluid [3] and serve as receptors for vascular endothelial growth factor (vEGF) [4] and fibroblast growth factor (FGF) [5]. The interaction between selectins and carbohydrate may be responsible for adhesion of inflammatory [6] or metastatic cells [7] to endothelium.

BAEC are heterogeneous and differ according to species, organ, and segment of the vascular tree [8]. Among these BAEC are the blood group antigens which are expressed in the endothelial cells in various vessels [9]. Studying vascular endothelia from primates, Ito *et al.* [10] suggested a close evolutionary relationship between the expression of blood group antigens and lectin binding properties of endothelial cells. In recent years blood group specific lectins have been reported as highly sensitive markers of vascular endothelium [9–12], and have been used as markers for tumours derived from endothelial cells [13].

The capillary supply of skeletal muscles has significance in terms of energy metabolism of the muscle fibres [14]. It is well documented that human skeletal muscle adapts to use by increasing the number of capillaries, and it appears that the capillaries begin to proliferate before changes are detectable in the oxidative enzymes of the muscle fibres [15]. Studies of the microvascularization of skeletal muscles is therefore of great importance in evaluating the muscle

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response to exercise physiology, such as endurance training, and in fibre-type atrophy caused by immobilization, arthropathies and myasthenia gravis.

In this paper we have compared the binding properties in muscle capillaries of blood group specific lectins and monoclonal antibodies reacting with blood group carbohydrate antigens belonging to type 1 or 2 chains. The investigation was performed on sections of the masseter muscle from humans and various animals (mouse, rat, rabbit, cat, dog, pig, cow, and monkey). The aim of the study was both to evaluate specific and reliable markers for muscle capillaries in man and various laboratory animals and to characterize the carbohydrate structures on endothelial cells that show affinity to lectins and antibodies.

Materials and methods

Muscle and blood grouping

The material consisted of specimens from human and animal masseter muscles. Biopsies from the anterior part of human masseter muscles were removed from 20 adult volunteers without known systemic disorders, who gave their informed consent. None of the biopsies contained signs of vascular disease, estimated from histological examination. ABO grouping was determined on erythrocytes by routine hemagglutination assays. The animal material consisted of masseter specimens from mouse (NMR1) (four), rat (Panum Wistar) (four), rabbit (Ssc:CPH) (three), cat (domestic) (three), dog (beagle) (three), pig (Danish Landrace/Yorkshire) (three), cow (Red Danish Landrace) (three), and monkey (*Macaca fascicularis*) (one). Immediately after removal the muscle specimens were frozen in isopentane cooled to −150 °C with liquid nitrogen, stored at −80 °C, and cut on a cryostat at −25 °C in 6 μm transverse sections.

Lectins and antibodies

The following biotinylated lectins were used in this study: GS I (*Griffonia simplicifolia* agglutinin; Kem-En-Tec, Denmark), GS I B4 (*Griffonia simplicifolia* isolelectin B4 agglutinin; Sigma, USA), DBA (*Dolichos biflorus*, Horse gram, agglutinin; Kem-En Tec), LTA (*Lotus tetragonolobus*, winged or asparagus pea; Sigma), UEA-1 (*Ulex europaeus* agglutinin; Kem-En-Tec). Five mouse monoconal antibodies reacting with known blood group carbohydrate antigens belonging to type 1 (Galβ1-3GlcNAcβ1-R) or type 2 (Galβ1-4GlcNAcβ1-R) chains were used (Type 1 structures include Le^a. Type 2 structures include H, A and Le^y). The specificity (Table 1) and references for generation for these antibodies have been described previously [16, 17].

Histochemical staining

Lectins. The lectin staining of the sections from animal masseter was performed as described in a previous paper, using unfixed sections and avidin alkaline phosphatase to visualize the binding sites of the biotinylated lectins [18]. The lectins were diluted to concentrations of 10–2 μg ml^{-1}. Other sections were immersed in 1% paraformaldehyde in (TBS) with 1 mM $CaCl_2$ for 5 min, and thereafter rinsed 3 × 5 min in TBS. Some of these sections were incubated with lectins; others were fixed oxidized in 0.5% periodic acid for 5 min and rinsed 3 × 5 min in TBS before they were incubated with the lectins.

Table 1. Specificity of lectins.

Lectin	*Sugar specificity*	*Blood group specificity*
GS-I	α-D-Gal, α-D-GalNAc	A, B
GS-I B4	α-D-Gal	B
DBA	α-D-GalNAc	A
UEA-I	α-L-Fuc	H
LTA	α-L-Fuc	H

Demonstration of lectin binding to sections of human masseter muscle was performed as follows. 1, Fixation of sections in cold acetone for 10 min. 2, Washing 2 × 5 min in washing buffer (TBS containing 1 mM each of $CaCl_2$, $MnCl_2$ and $MgCl_2$). 3, Blocking of endogenous biotin by incubation with ExtrAvidin (Sigma) 1:100 in washing buffer 30 min. 4, Washing 3 × 5 min in the washing buffer. 5, Incubation overnight at 4 °C with the lectins diluted 1:200 in the washing buffer. 6, Rinsing 3 × 5 min in TBS. 7, Incubation for 30 min at room temperature with alkaline phosphatase-conjugated avidin (DAKOPATTS D 365; Denmark) diluted 1:300 with TBS, pH 7.6, 0.1 M). 8, Washing in TBS, pH 7.2, 0.1 M, 3 × 5 min. 9, Incubation for 20–30 min at room temperature in a medium containing 10 ml Tris buffer, pH 9.5, 0.1 M, 10 mM $MgCl_2$, 2.5 mg 5-bromo-4-chloroindoxyl phosphate (Bachem, Switzerland) in 0.1 ml *N*,*N*-dimethylformamide, 3 mg Nitro Blue tetrazolium (NBT, Sigma), 2 mg levamisole (Sigma).

Monoclonal antibodies. Unfixed sections were: 1, incubated with the primary antibodies overnight at 4 °C; 2, washed 3 × 5 min in TBS; 3, incubated 30 min at room temperature with alkaline phosphatase-conjugated mouse immunoglobulins (DAKOPATTS D 314) diluted 1:200 with TBS, pH 7.6, 0.1 M; 4, the subsequent washing and incubation in the indoxyl phosphate medium was as above. Other sections were immersed in 1% paraformaldehyde in TBS with 1 mM $CaCl_2$ for 5 min and thereafter rinsed 3 × 5 min in TBS. Some of these sections were incubated with antibodies, others were oxidized in 0.5% periodic acid for 5 min and rinsed 3 × 5 min in TBS before they were incubated with the antibodies.

In control sections, incubation was performed without the primary antibody or lectin. Some sections were incubated with the culture supernatant used for hybridization.

We have not used monosaccharides as inhibitor controls for the lectin staining since lectins often show affinities for a number of different carbohydrates and sequences of carbohydrates which render such inhibition studies of limited value [19].

Results and discussion

Animals

Lectin binding to muscle capillaries showed great variability between the species, whereas the capillaries from individual animals within the same species showed identical reactions. The lectin reactions are summarized as follows. *UEA-I*: No capillary reaction in any muscle section. *DBA*: No capillary reaction in any muscle section.

GS I: This lectin is composed of two distinct subunits, A and B [20]. A strong reaction in sarcolemma and interfibre connective tissue impedes the identification of muscle capillaries in cow, dog, cat, monkey, pig, and rabbit (Fig. 1). In rat capillaries there was a strong capillary affinity to GS I and a weak GS I binding to the sarcolemma and interfibre connective tissue (Fig. 2). In mouse masseter muscle the capillaries are the only structures with affinity to GS I (Fig. 3). *GS I B4*: In cow, dog, cat, monkey, pig, and rabbit the staining reaction with this isolectin was similar to the staining pattern with GS I (Fig. 4). However, GS I B4 clearly marked capillaries in mouse and rat muscles (Fig. 5). GS I is a tetrameric lectin with affinity for terminal galactose and *N*-acetylgalactosamine, while the isolectin GS I B4 has exclusive affinity for terminal galactose. GS I has previously been used as a specific capillary marker for capillaries in rat muscle [12, 21]. In this study, incubation with GS I strongly stained rat muscle capillaries, but we also noticed staining in the sarcolemma and connective tissue. The discrepancies between these observations could be due to variations of endothelial cell glycosylation between different strains of rats. Thus, in the animal muscles used in this study, GS I and GS I B4 can be recommended only as specific markers for capillaries in mouse and rat.

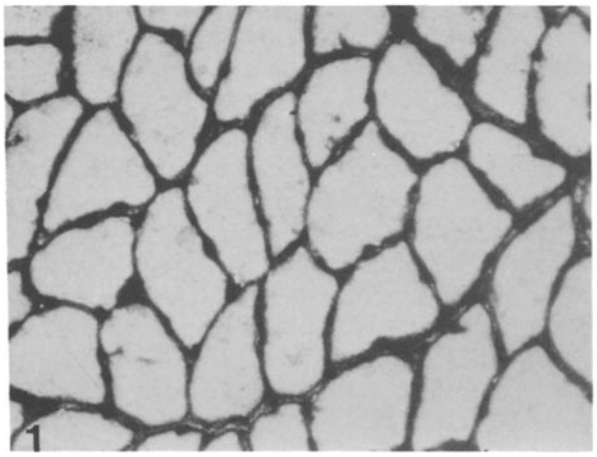


Figure 1. GS I staining of rabbit masseter muscle. The capillaries are difficult to identify due to the strong staining of the muscle fibre periphery (× 252).

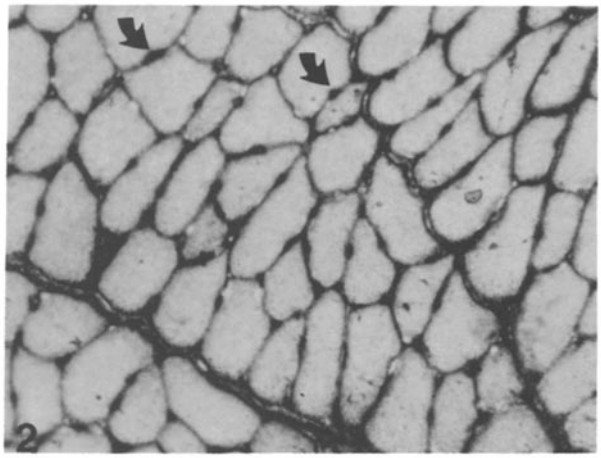


Figure 2. GS I staining of rat masseter muscle. The lectin staining of the capillaries is stronger than the staining of the sarcolemma and connective tissue. Two capillaries are marked by arrows (× 252).

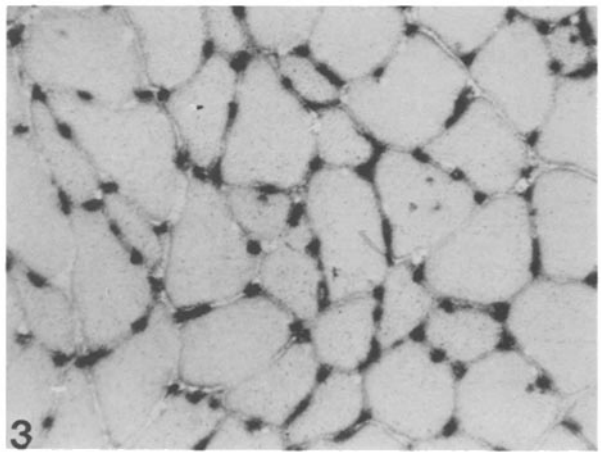


Figure 3. GS I staining of mouse masseter muscle. The capillary reaction is strong while there is no staining in the sarcolemma or interfibre connective tissue (× 252).

LTA: In all muscles incubation with LTA resulted in a weak, diffuse reaction often most obvious around vascular structures (Fig. 6). Formaldehyde fixation of the muscle sections depressed the LTA staining, whereas in formaldehyde fixed, periodic acid oxidized sections there was a strong, localized capillary reaction after incubation with LTA in all muscles examined except *Macaca* muscles. In sections from mouse, rat and pig masseter muscles it appears that the capillaries were the only structures with affinity for LTA (Fig. 7). In cat, dog, cow, and rabbit also a weak sarcolemmal reaction could be noticed. In formaldehyde–periodic acid treated sections from *Macaca* masseter muscle LTA incubation resulted in moderate staining of capillaries,

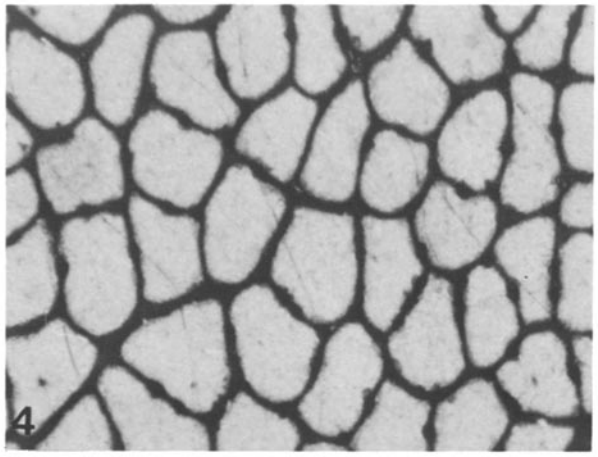


Figure 4. GS I B4 staining in rabbit masseter muscle. The muscle fibre periphery and the interfibre connective tissue are strongly stained (× 252).

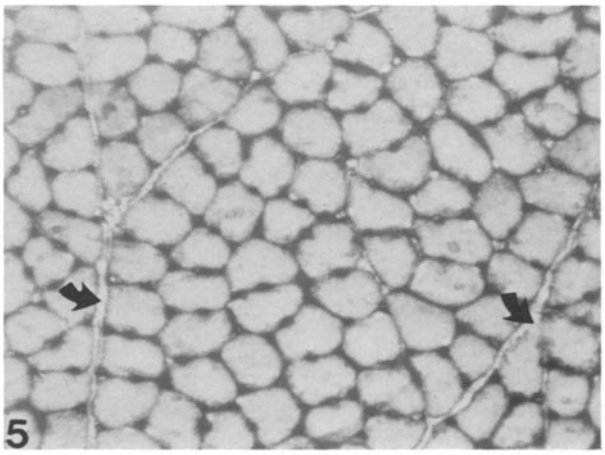


Figure 5. GS I B4 staining in rat masseter muscle. The capillaries are clearly expressed due to the weak sarcolemmal reaction and the lack of staining in the connective tissue septa (arrows) (× 252).

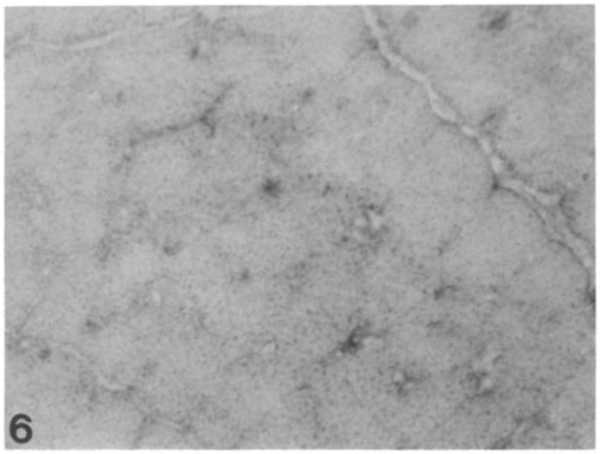


Figure 6. LTA staining of pig masseter muscle. The reaction is weak and diffuse (× 252).

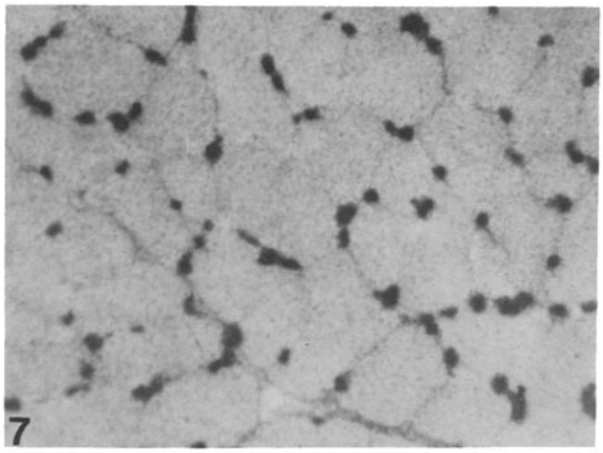


Figure 7. LTA staining of a pig masseter muscle section which has been treated with formaldehyde and thereafter with periodic acid before lectin incubation. Note the strong, selective capillary reaction (× 252).

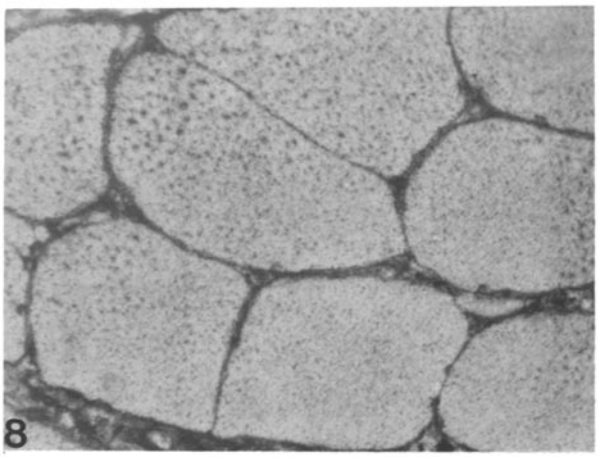


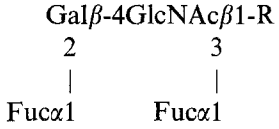
Figure 8. LTA staining of a *Macaca* masseter muscle section which has been treated with formaldehyde and thereafter with periodic acid before lectin incubation. Note the reaction in the sarcolemma, connective tissue and capillaries (× 252).

sarcolemma and connective tissue (Fig. 8). All micrographs shown in this paper are of the same magnification and thus reflect the great interspecies variation in the size of muscle fibres in the masseter. LTA has not been much used as a marker for vascular structures. Roussel and Dalion [22] found no LTA binding in endothelial cells from mouse, rat, rabbit, cat, dog, cow, pig, and *Macaca mulatta*. Griffiths and Stephenson [23], demonstrated some LTA binding to vascular endothelium in follicular carcinoma of the thyroid, while Holthöfer *et al.* [9] were unable to detect LTA reaction with endothelial cells from various human tissues. An expansion of the conflicting results could be differences in tissue handling, since extraction of demonstrable fucose-containing glycoconjugates occurs during routine fixation

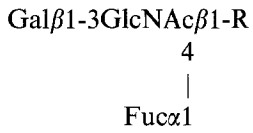
and paraffin wax processing [23]. We observed that the weak LTA reaction around vascular structures in unfixed sections was totally inhibited after short formaldehyde fixation.

Periodic acid is used as an oxidizing agent in the periodic acid–Schiff (PAS) reaction since carbohydrate C–C bonds having vicinal –OH groups (1,2-diol groups) will be cleaved by periodic acid, converting them into Schiff reactive carbonyl groups. The carbohydrate structure in capillaries that is positive for LTA binding following a brief fixation and periodic acid treatment may thus, it its non-oxidized condition, be hidden by accretion of external carbohydrate that masks the LTA binding glyco compound.

Different fucosylated oligosaccharides have been proposed to show sugar-binding specificities for LTA. Using quantitative precipitation-inhibition assays, Sugii and Kabat [24] found that the most specific carbohydrate for LTA was an oligosaccharide with the structure:



i.e., type 2 chain Le^y determinant, while Spicer and Schulte [25] suggest that LTA binds to Fucα1-4GlcNAc in both Le^a and Le^b haptens. However, incubation with antibodies against type 2 chain Le^y (above) and type 1 chain Le^a



did not show capillary staining in any muscle section treated with periodic acid. By use of synthetic glycoproteins and oligosaccharides, Petryniak and Goldstein [26] likewise noted that the Le^a determinant did not precipitate with lotus lectin. Even if at present we are unable to determine the structure of the fucose-containing carbohydrate in muscle capillaries that shows affinity to LTA after periodic acid treatment, this procedure might be useful to demonstrate muscle capillaries in animals when other lectin methods fail.

Humans

The material consisted of eight blood group O, nine blood group A, two blood group B, and one blood group AB individuals. Positive capillary reaction was noticed in muscle capillaries with H type 2 antigen and the lectins UEA-1, GS-I, GS-I B4 and DBA. No reaction was found in the muscle capillary endothelial cells after incubation for Le^a, A type 2 antigen or Le^y. The results are summarized in Table 2.

Based on staining with UEA, the capillaries in all the biopsies were stained but with different intensities. In one group of biopsies the endothelial cells were strongly stained with UEA-I. These biopsies belong to blood group O, B, or AB. In the other group the endothelial cells reacted weakly. The biopsies from these patients were from blood group A donors. The results in Table 2 further indicate that only the muscle capillaries from O individuals were stained with antibody against the blood group H type 2 determinant. Table 2 also shows that GS-I strongly stains muscle capillaries in A, B, and AB individuals while GS-I B4 marked the capillaries in B and AB donors only. Incubation with GS-I B4 also resulted in a moderate sarcoplasmic staining in most muscle fibres. Finally, a weak capillary reaction with DBA was noted in A individuals. In Figs 9–17 the muscle capillary reaction in O donors (Figs 9–11), A donors (Figs 12–14) and B donors (Figs 15–17) are shown. Control sections showed no capillary reaction.

Table 2. Capillary staining after incubation with lectins and anti-H type 2 antibody in individuals with different blood groups.[a]

No. of cases	*Blood group*	*H-antigen*	*UEA-I*	*GS-I*	*GS-I B4*	*DBA*
8	0	++	++	0	0	0
9	A	0	+	++	0	(+)[b]
2	B	0	++	++	++	0
1	AB	0	++	++	++	(+)

[a] ++, strong capillary reaction; +, moderate capillary reaction; (+), weak capillary reaction; 0, no reaction.
[b] In one biopsy only a few endothelial cells were marked after DBA incubation.

Although GS I is believed to be specific for determinants A and B and GS I B4 for B in human tissue [27], previous attempts to visualize human endothelial cells after incubation with these lectins have not been successful. Endothelial staining after GS I B4 incubation was described as negative by Peters and Goldstein [28] and Alroy *et al.* [29]. Ito *et al.* [26] have compared staining with blood group-specific lectins and monoclonal antibodies to recognize the ABH

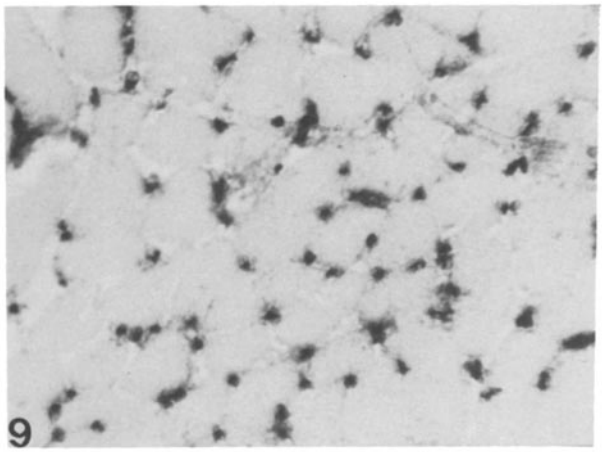


Figure 9. UEA I staining in blood group O individual. There is strong activity in muscle capillaries (×252).

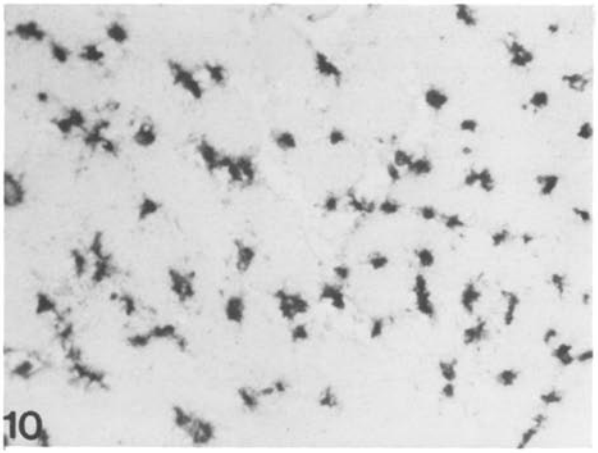


Figure 10. UEA I staining after incubation with monoclonal antibody against H antigen in blood group O individual. There is strong activity in muscle capillaries (×252).

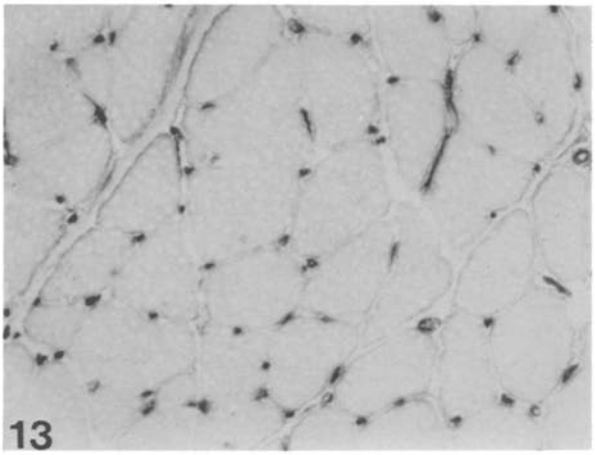


Figure 13. GS I staining in blood group A individual. There is strong activity in muscle capillaries (×252).

Figure 11. GS I staining in blood group O individual. There is virtually no reaction in the capillaries (×252).

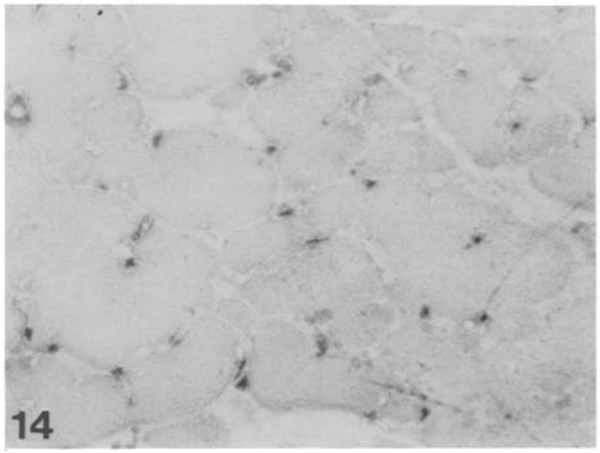


Figure 14. DBA staining in blood group A individual. The capillary reaction is weak (×252).

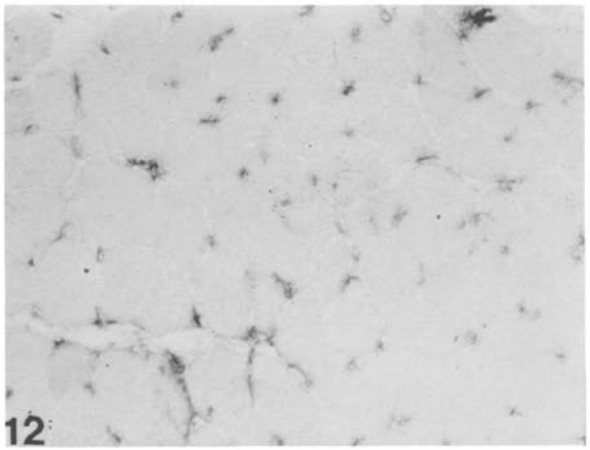


Figure 12. UEA I staining in blood group A individual. The capillary reaction is only moderate (×252).

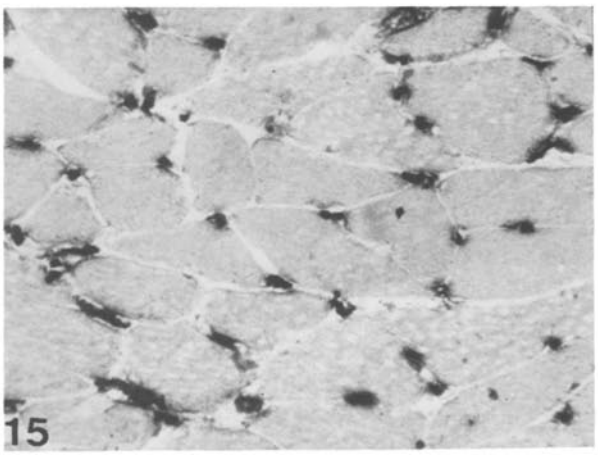


Figure 15. UEA I staining in blood group B individual. There is strong activity in muscle capillaries (×252).

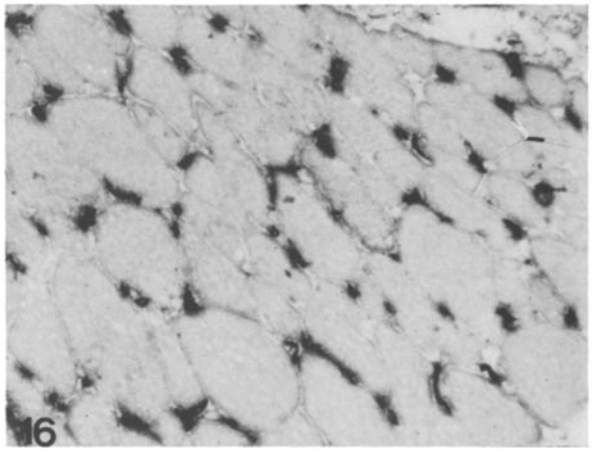


Figure 16. GS I staining in blood group B individual. There is strong activity in muscle capillaries (× 252).

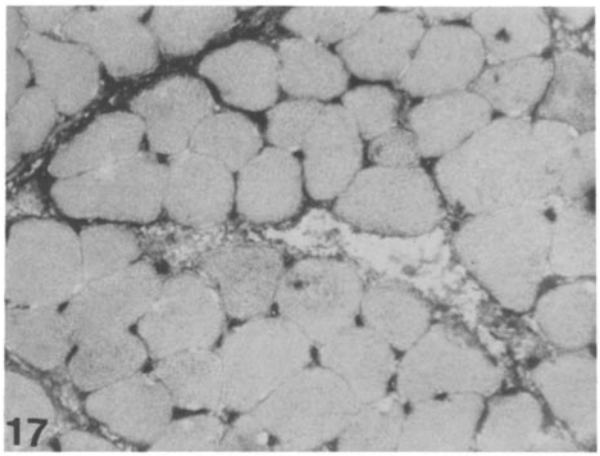


Figure 17. GS I B4 staining in blood group B individual. There is strong activity in muscle capillaries. Note that strains of connective tissue are also stained (× 252).

antigens in human tissues. They did not note reactivity with GS I or GS I B4 in endothelial cells from A, B, or AB donors. Using a sensitive alkaline phosphatase conjugated avidin method to demonstrate binding of biotinylated lectins [18] we could detect strong capillary staining in muscles from A, B, and AB donors with GS I and in muscles from B and AB donors with GS I B4, thus confirming biochemical evidence concerning the specificity of these lectins.

It has been shown previously that the glycoconjugates of human vascular endothelium contain fucose residues which specifically bind to UEA-I [9] while endothelial cells of animal origin do not contain UEA-I receptors [21]. UEA-I is proposed to stain human vascular endothelium regardless of blood group [29] and the carbohydrate with specific affinity to UEA-I is believed to be the H-type 2 monofucosyl oligosaccharide with the structure [30]:

$$\begin{array}{l} \text{Gal}\beta\text{1-4GlcNAc}\beta\text{-R} \\ \quad\;\; 2 \\ \quad\;\; | \\ \text{Fuc}\alpha\text{1} \end{array}$$

We have found that in all human biopsies the capillaries were stained after incubation with UEA-I but in such a way that the endothelial cell reaction from blood group A donors was much weaker than the reaction in O, B, and AB donors. This agrees with the greater ability of the A-enzyme to transfer the A-determinant to the H-substrate than that of the B-enzyme to transfer the B-determinant. The monoclonal antibody detecting the above carbohydrate structure marked muscle capillaries only in tissue from O donors. Thus monoclonal antibody against the H-type 2 antigen, which is a precursor to A and B, showed no affinity for muscle capillaries from A, B, or AB individuals in agreement with the notion that the H-antigen is more strongly expressed in O-individuals than A- and B individuals. The apparent discrepancy between staining with UEA-I and the anti-H antibody is most likely a result of the more restricted specificity of the monoclonal antibodies compared with lectins.

DBA is anti-A and thus has affinity for terminal *N*-acetylgalactosamine [31]. The endothelial cell staining with DBA differs according to species, organ and segment of the vascular tree [24]. In this study none of the animal species showed muscle capillary staining after incubation with DBA whereas, in human muscle, staining was noted in capillaries from all blood group A donors. This is in contrast to the results of Ito *et al.* [27] who did not observe DBA endothelial staining in human tissues from blood group A. In one biopsy from an A individual only a few capillaries were marked with DBA. This difference in endothelial A-antigen expression between A donors could be due to different A-subgroups.

In the control sections no staining was observed. Thus, the endogenous alkaline phosphatase in the capillaries is completely blocked by levamisole.

We have shown that capillaries from human skeletal muscle contain carbohydrate compounds that reflect the individual blood type. They can be identified clearly in all blood types if two muscle sections are incubated with GS I and UEA I, respectively, whereby the microvascular density in skeletal muscle may be estimated. Different lectins were shown to be suitable for demonstrating muscle capillaries in a number of laboratory animals.

Acknowledgements

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