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DISTRIBUTION OF FERRITIN-CONJUGATED LECTINS ON SIALIDASE-TREATED MEMBRANES OF HUMAN ERYTHROCYTES

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SUMMARY

The labeling of sialidase-treated, human erythrocyte membranes with ferritin-conjugates of four plant lectins, concanavalin A, *Ricinus communis* hemagglutinin, *Bauhinia purpurea* hemagglutinin and *Arachis hypogoea* hemagglutinin, is reported. Among these ferritin-conjugated lectins, ferritin-conjugated concanavalin A and ferritin-conjugated *R. communis* hemagglutinin were found in clusters on the sialidase-treated membranes, whereas ferritin-conjugated *B. purpurea* hemagglutinin and ferritin-conjugated *A. hypogoea* hemagglutinin were found in a random distribution on the membranes. Furthermore, when the membranes were labeled with a mixture of concanavalin A and ferritin-conjugated *B. purpurea* hemagglutinin, ferritin particles were found in clusters, indicating that the membrane receptors for *B. purpurea* hemagglutinin were forced to move together with those for concanavalin A. A method for the quantitative estimation of the clustering of ferritin particles on the membranes was also devised and applied to the labeling of sialidase-treated, human erythrocyte membranes with the above four ferritin-conjugated lectins.

INTRODUCTION

In the previous papers [1–3], we have classified several plant lectins into three groups based on their sugar-binding specificities, shown by the hemagglutination-inhibition assays using glycoproteins [1] and glycopeptides [2] as hapten inhibitors, and by the competitive binding of the ^{131}I-labeled lectins to human erythrocytes [3]. The lectins of the first group, which includes *Bauhinia purpurea* and *Arachis hypogoea* hemagglutinins, bind primarily to the cell-surface sugar chains having a Gal → GalNAc sugar sequence such as the *O*-glycosidically linked sugar chain of major sialoglycoprotein of human erythrocyte membrane, whereas those of the second group, which includes concanavalin A and *Ricinus communis* hemagglutinin, bind

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preferentially to the cell-surface sugar chains having a Gal → GlcNAc sugar sequence such as the *N*-glycosidically linked sugar chain of major sialoglycoprotein and, presumably, of Band III glycoprotein (Kawaguchi, T. and Osawa, T., unpublished observations) of the human erythrocyte membrane. The lectins of the third group, which includes soybean, *Wistaria floribunda* and *Sophora japonica* hemagglutinins, can probably bind to either type of sugar chain.

This paper presents evidence that lectins which differ in their sugar-binding specificities give rise to different topographic distribution of membrane receptors on the outer surface of the human erythrocyte, and that different lectin receptors may be structurally linked in the human erythrocyte membrane.

MATERIALS AND METHODS

Ferritin. Horse spleen ferritin (six times recrystallized, cadmium-free) was obtained from Miles Laboratories (Elkhart, Ind., U.S.A.), and was further purified by crystallization in the presence of cadmium sulfate and precipitation at 50 % ammonium sulfate.

Lectins. Concanavalin A was purified from jack bean meal (Sigma, St. Louis, Mo., U.S.A.) according to the method of Agrawal and Goldstein [4]. *R. communis* hemagglutinin and *B. purpurea* hemagglutinin were purified by the methods previously described [5, 6]. *A. hypogoea* (peanut) hemagglutinin was purified by affinity chromatography according to the following method which will be described in detail elsewhere. The crude extract of the *A. hypogoea* seeds was first fractionated with $(NH_4)_2SO_4$. The fraction which precipitated between 0.30 and 0.75 saturation of $(NH_4)_2SO_4$ was further subjected to affinity chromatography on a Sepharose 6B column.

Enzymes. Clostridium perfringens neuraminidase was purchased from Böhringer-Mannheim Biochemicals (Mannheim, G.F.R.), trypsin (twice recrystallized, code TRL) from Worthington Biochemical Co. (Freehold, N. J., U.S.A.).

Cells. Human group OMN venous blood was withdrawn into syringes previously treated with heparin. The heparinized blood was transferred to glass cylinders, and the erythrocytes were allowed to sediment by gravity. The erythrocyte layer, after removal of leukocyte-rich plasma and buffy coat, was washed three times with 5 mM phosphate buffered saline (pH 7.1). Enzyme treatments of the erythrocytes were carried out under the conditions previously described [6].

Preparation of desialized porcine submaxillary mucin-Sepharose 4B. 100 mg of porcine submaxillary mucin purified from porcine submaxillary glands showing blood group A activity according to the method of Katzman and Eylar [7] was coupled to CNBr-activated Sepharose 4B by the method previously described [8]. The adsorbant thus prepared was incubated with 0.1 unit of *C. perfringens* neuraminidase in 0.1 M sodium phosphate buffer (pH 6.0) containing 0.2 % bovine serum albumin at 37 °C for 2 h.

Ferritin-conjugated lectins. The affinity-purified lectins were conjugated to ferritin by a modification of the glutaraldehyde coupling procedure of Avrameas [9]. To a solution of 5 mg of lectin and 50 mg of the purified ferritin in 2 ml of 0.1 M sodium phosphate buffer (pH 7.2) was carefully added, with stirring, 1.0 % glutaraldehyde to a final concentration of 0.05 %, and the mixture was incubated at room

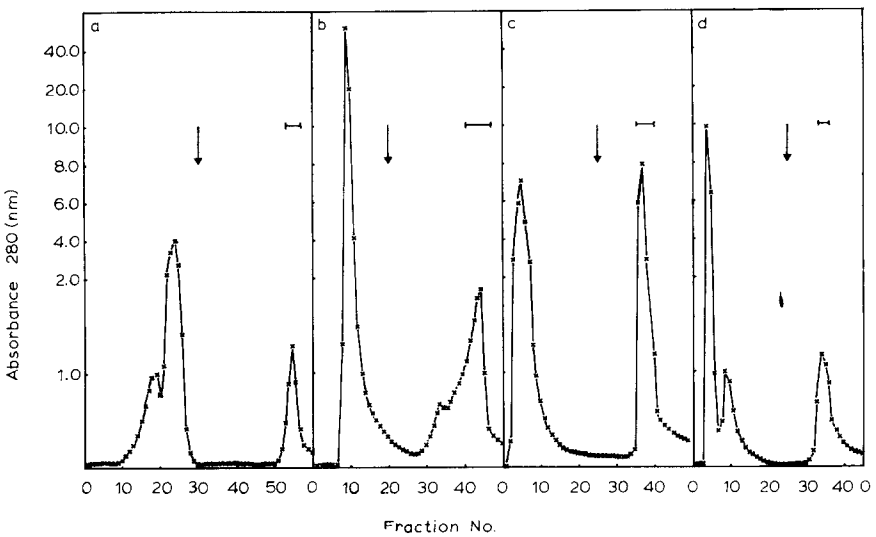

Fig. 1. (a) Affinity chromatography of ferritin-*R. communis* hemagglutinin on Sepharose 4B. The conjugation mixture (2 ml) was applied to a column (5×28 cm) previously equilibrated with 5 mM phosphate buffered saline (pH 7.1), and eluted with the same buffer. After inactive proteins were eluted, the column was further eluted (arrow) with the same buffer containing 0.2 M lactose. Fractions of 14 ml were collected at 30 ml per h at 4 °C. Fractions indicated by a double-headed arrow were combined and dialyzed against the phosphate buffered saline. (b) Affinity chromatography of ferritin-concanavalin A on Sephadex G-100. The conjugation mixture (2 ml) was applied to a column (2×40 cm) previously equilibrated with 5 mM phosphate buffered saline, and the column was eluted with the same buffer. After elution of inactive proteins, the column was further eluted (arrow) with the same buffer containing 0.1 M D-glucose. Fractions of 4.5 ml were collected at 20 ml per h at 4 °C. The fractions indicated by a double-headed arrow were combined and dialyzed against the phosphate buffered saline. (c) Affinity chromatography of ferritin-*B. purpurea* hemagglutinin on desialized porcine submaxillary mucin-Sepharose 4B. The conjugation mixture (2 ml) was applied to a column (1.5×12 cm) and the column was eluted with 5 mM phosphate buffered saline (pH 7.1). After elution of inactive proteins, the column was eluted (arrow) with the same buffer containing 0.2 M lactose. Fractions of 5 ml were collected at 25 ml per h at 4 °C. The fractions indicated by a double-headed arrow were combined and dialyzed against the phosphate buffered saline. (d) Affinity chromatography of ferritin-*A. hypogoea* hemagglutinin on desialized porcine submaxillary mucin-Sepharose 4B. The conjugation mixture (1 ml) was applied to the same column as that for ferritin-*B. purpurea* hemagglutinin. The elution of the active protein was performed in the same fashion as for ferritin *B. purpurea* hemagglutinin.

temperature for 1 h. The reaction mixture was then dialyzed against 5 mM phosphate buffered saline (pH 7.1) to remove free glutaraldehyde. The ferritin-conjugated lectin was separated from free ferritin and also from the inactivated lectin by appropriate affinity chromatography. The specific adsorbants used for affinity chromatography were Sephadex G-100 for ferritin-conjugated concanavalin A, and Sepharose 4B for ferritin-conjugated *R. communis* hemagglutinin. Desialized porcine submaxillary mucin-Sepharose 4B was used for the specific purification of ferritin-conjugated *B. purpurea* hemagglutinin and ferritin-conjugated *A. hypogoea* hemagglutinin. The elution of the active ferritin-lectins was carried out with 5 mM phosphate buffered saline (pH 7.1) containing 0.1 M D-glucose for ferritin-concanavalin A, and with the phosphate buffered saline containing 0.2 M lactose for ferritin-*R. communis* hemag-

TABLE I

CHEMICAL ANALYSIS AND HEMAGGLUTINATING ACTIVITY OF FERRITIN-LECTINS

	Ferritin/Lectin		Minimum hemagglutinating dose (μg lectin/ml)	
	mg/mg	molar ratio		
Ferritin-concanavalin A	16.0	2.4	3.8[a]	> 120[b]
Ferritin-*R. communis* hemagglutinin	10.6	1.6	2.0[b]	
Ferritin-*B. purpurea* hemagglutinin	5.0	1.3	0.3[b]	
Ferritin-*A. hypogoea* hemagglutinin	11.5	1.6	6.7[b]	
Concanavalin A			0.7[a]	250[b]
R. communis hemagglutinin			0.8[b]	
B. purpurea hemagglutinin			0.2[b]	
A. hypogoea hemagglutinin			3.9[b]	

[a] Against guinea pig erythrocytes.
[b] Against sialidase-treated human erythrocytes.

glutinin, ferritin-*B. purpurea* hemagglutinin, and ferritin-*A. hypogoea* hemagglutinin as shown in Fig. 1. The fractions containing ferritin-lectin were extensively dialyzed against the phosphate buffered saline and concentrated by ultrafiltration. Polymerized conjugates were removed by centrifugation at 30 000 $\times g$ for 20 min before use. This procedure for the labeling of lectins with ferritin did not significantly affect the hemagglutinating activity of the lectins used in this study, as shown in Table I.

Disc electrophoresis. Purity of the ferritin-lectins was examined by polyacrylamide disc electrophoresis in 3.75 % gels in 0.1 M phosphate buffer at pH 7.2. Staining was performed with Coomassie Brilliant Blue R in 7 % acetic acid; destaining was carried out with 5 % methanol containing 7.5 % acetic acid.

Chemical analysis of ferritin-lectins. Protein was determined by the method of Lowry et al. [10]. Iron was determined by an *o*-phenanthroline method according to Matsubara [11].

Ferritin-lectin labeling. Ferritin-lectin labeling was performed on sialidase-treated human erythrocyte membranes according to the method of Nicolson and Singer [12]. A small volume of a 10 % suspension of erythrocytes in 5 mM phosphate buffered saline (pH 7.1) was dropped onto a surface of distilled water. Under these conditions, a small fraction of the erythrocytes ruptured and remained at the air-water interphase. These flattened erythrocyte membranes were picked up on a carbon-strengthened colloidon membrane on a copper grid. The grids with the attached membranes were coated with 15 % bovine serum albumin for 15 min to eliminate the non-specific adsorption of the ferritin-lectin onto the collodion membrane and the erythrocyte membranes. Before being dried, the grid was stained with a 0.1 % solution of ferritin-lectin at 37 °C for 30 min. Then the grid was washed by floating it on 10 consecutive drops of distilled water. After being dried, the specimen was observed by transmission electron microscopy in a JEOL model JEM-100 B electron microscope.

Quantitative representation of a ferritin distribution. On the electron microscopic photograph, regular squares with a 9000 Å side length were divided into 36 $\times$ 36 (1296) parts, and the number of ferritin particles in each small square with a 250 Å

side length (unit area) was counted. If we assume a matrix $(X_{p,q})$:

$$X_{p,q} = \begin{pmatrix} X_{1,1}, & X_{1,2} \cdots X_{1,36} \\ X_{2,1}, & X_{2,2} \cdots X_{2,36} \\ & \\ X_{36,1}, & X_{36,2} \cdots X_{36,36} \end{pmatrix}$$

the average number of the ferritin particles in an unit area is

$$\frac{1}{1296} \sum_{p,q=1}^{36} X_{p,q}$$

The standard deviation (σ) of the number of the ferritin particles in the regular squares with a 250 Å side length is then calculated by the equation:

$$\sigma = \sqrt{\frac{1}{1296} \sum_{i,j=1}^{36} \left(X_{p,q} - \sum_{i,j=1}^{36} X_{p,q} \right)} \tag{1}$$

Similarly, the standard deviation of the number of the ferritin particles in the regular squares with a $250 \times k$ Å side length is shown by the equation:

$$\sigma(k) = \sqrt{\frac{1}{(37-k)^2} \sum_{i,j=1}^{37-k} \left(\frac{1}{k^2} \sum_{p,q=i,j}^{i=k-1} X_{p,q} - \sum_{i,j=1}^{36} X_{p,q} \right)} \tag{2}$$

On the other hand, two-dimensional random distribution was prepared for various numbers of ferritin particles (200–800) from a table of random numbers. The values of $\sigma_{\text{random}}(k)$ were calculated from this distribution and equation (2) as shown in Fig. 2. These values are functions of a variable k and of the total number of ferritin particles present, and can be approximated by use of the equation:

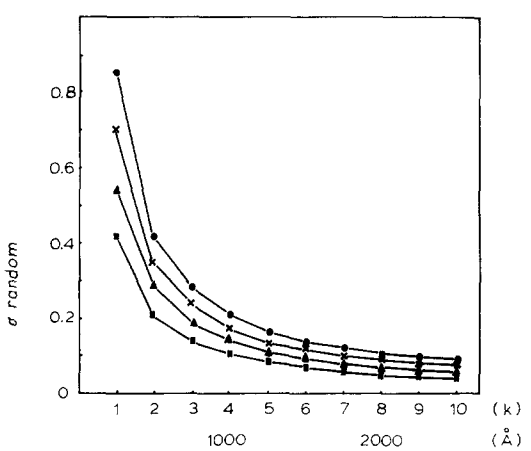

Fig. 2. Standard deviation of the number of ferritin particles in random distribution. The random distribution of various numbers of ferritin particles (-■-, 200; -▲-, 400; -x-, 600; -●-, 800) in 1296 squares are prepared from a table of random numbers. σ values were calculated from this distribution and equation (2) in Materials and Methods and illustrated against various k values.

$$\sigma_{\text{random}}(k) = 0.029\sqrt{TN}/k \qquad (k < 6) \tag{3}$$

where TN is the total number of the ferritin particles in 1296 unit areas and 0.029 is a constant obtained from Fig. 2. If the ferritin particles bound to the cell-surface membrane form clusters, the $\sigma(k)$ value increases. We therefore introduced the ratio $b(k)/\sigma_{\text{random}}(k)$ as a parameter of clustering.

RESULTS

Properties of purified ferritin-lectins. The purified ferritin-lectins after centrifugation at 30 000 × *g* showed one or two diffuse bands on examination by polyacrylamide gel electrophoresis in 3.75 % gels at pH 7.2. No free lectin was detected. When dilute ferritin-lectin solutions sprayed on the coated grid were examined, most of the ferritin in these solutions was present as small molecular species (ferritin/lectin = 1 : 1 or 2 : 1) as shown in Fig. 3(a). Furthermore, the results of the determination of iron and protein in the ferritin-lectins revealed that the ratio of ferritin to lectin was less than 3 (Table I).

Two-dimensional distribution of ferritin-lectins on human erythrocyte membrane. When intact human erythrocyte membranes were labeled with each ferritin-lectin, the amount of the ferritin-lectin bound was too small to evaluate its distribution, particularly for ferritin-*B. purpurea* hemagglutinin and ferritin-*A. hypogoea* hemagglutinin. However, even in this case, the distribution of ferritin-concanavalin A and ferritin-*R. communis* hemagglutinin was apparently different from that of ferritin-*B. purpurea* hemagglutinin, in that ferritin particles tended to segregate in forming small clusters. This pattern was more apparent for sialidase-treated human erythrocyte membranes. Fig. 3 shows the distribution of ferritin-concanavalin A, ferritin-*R.communis* hemagglutinin, ferritin-*B. purpurea* hemagglutinin and ferritin-*A. hypogoea* hemagglutinin on human erythrocyte membranes which had been treated with sialidase. In Fig. 3(d) and Fig. 3(e), ferritin-*B. purpurea* hemagglutinin and ferritin-*A. hypogoea* hemagglutinin appeared to be dispersed as single particles, and the formation of small clusters could not be observed. On the contrary, as shown in Fig. 3(b) and Fig. 3(c), ferritin-concanavalin A and ferritin-*R. communis* hemagglutinin were distributed in small clusters, possibly due to cross-linkage of receptor molecules. Since the amount of each ferritin-lectin bound to the sialidase-treated membranes was of almost the same order when the same dose of the ferritin-lectins was applied, the degree of clustering may not be simply a function of the amount of lectin bound.

Scale and degree of clustering. Fig. 4 shows the ratio $\sigma(k)/\sigma_{\text{random}}(k)$ for the binding of each ferritin-lectin on the sialidase-treated membranes of human erythrocytes. From the curve shown, the diameter of most of the clusters formed by ferritin-*R. communis* hemagglutinin and ferritin-concanavalin A was estimated as about 1000 Å. Ferritin-*B. purpurea* hemagglutinin and ferritin-*A. hypogoea* hemagglutinin rarely formed clusters. Since the diameter of a single ferritin-lectin molecule is about 100–150 Å at its maximum, this observation suggests that the clustered distribution of ferritin-concanavalin A and ferritin-*R. communis* hemagglutinin results from the cross-linkage of their receptor molecules in the membranes.

Alteration of the distribution of ferritin-B. purpurea hemagglutinin by concanavalin A. It is possible that the difference in the distribution pattern of the ferritin-

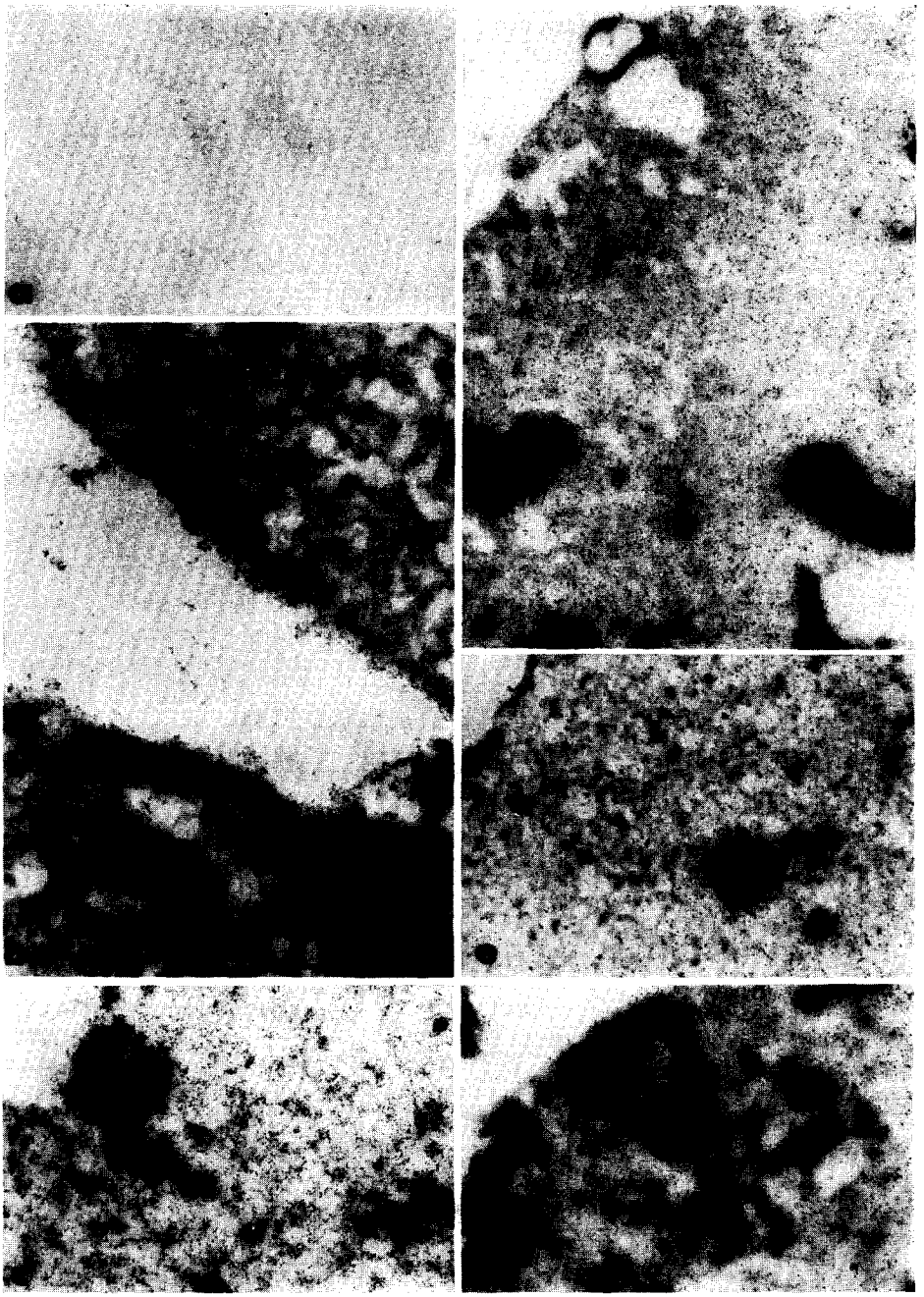

Fig. 3. Electron microscopic photographs. (a) Ferritin-concanavalin A dried down on a carbon-collodion film (×22 000). (b) Sialidase-treated, human erythrocyte membrane labeled with ferritin-*R. communis* hemagglutinin (×22 000). (c) Sialidase-treated, human erythrocyte membrane labeled with ferritin-concanavalin A (×22 000). (d) Sialidase-treated, human erythrocyte membrane labeled with ferritin-*B. purpurea* hemagglutinin (×22 000). (e) Sialidase-treated, human erythrocyte membrane labeled with ferritin-*A. hypogoea* hemagglutinin (×22 000). (f) Sialidase-treated, human erythrocyte membrane labeled with a mixture of concanavalin A and ferritin-*B. purpurea* hemagglutinin (×22 000).

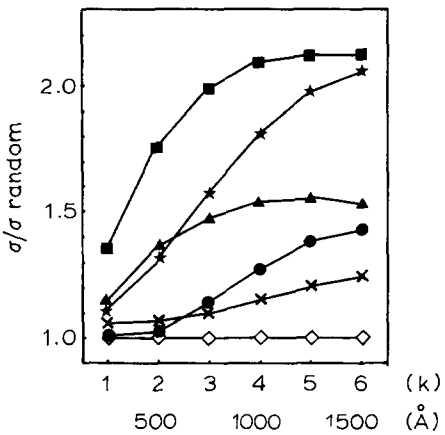

Fig. 4. The parameter of clustering of ferritin particles (σ/σ_{random}) calculated for sialidase-treated, human erythrocyte membranes labeled with various ferritin-lectins. The calculation of the parameter was carried out as described in the text. -■-, ferritin-*R. communis* hemagglutinin; -▲-, ferritin-concanavalin A; -●-, ferritin-*B. purpurea* hemagglutinin; -×-, ferritin-*A. hypogoea* hemagglutinin; -★-, ferritin-*B. purpurea* hemagglutinin+concanavalin A; -◇-, random distribution.

lectins bound to the membranes depends on the original distribution pattern of their receptors on the membranes. To examine whether this is the case, an attempt was made to label the sialidase-treated, human erythrocyte membranes with ferritin-*B. purpurea* hemagglutinin mixed with 0.2 % concanavalin A solution. On electron microscopic examination, the ferritin particles formed clusters as shown in Fig. 3(f). The values of $\sigma(k)/\sigma_{random}(k)$ observed in this experiment were compared with the values for ferritin-*B. purpurea* hemagglutinin and ferritin-concanavalin A in Fig. 4. This indicated that the receptors for ferritin-*B. purpurea* hemagglutinin were forced to move together with the receptors for concanavalin A which were crosslinked with unlabeled concanavalin A. Fig. 4 also shows that ferritin-*B. purpurea* hemagglutinin tends to distribute somewhat unevenly on the membrane, because $\sigma(k)/\sigma_{random}(k)$ value for the binding of ferritin-*B. purpurea* hemagglutinin increases in the squares with a side length of longer than 1000 Å.

DISCUSSION

Ferritin-conjugated plant lectins have been used extensively as specific electron microscope stains for sugar moieties on the membranes of normal and transformed cells [12–15]. In the present study, the ferritin-lectins were purified by means of affinity chromatography to remove unconjugated proteins and inactivated conjugates in the conjugation mixture. Since these purified conjugates retained almost the same hemagglutinating activity as the intact lectins and contained only one to two ferritin molecules per molecule of lectin, they were considered to be satisfactory even for the quantitative investigation of the distribution of lectins on biological membranes. Furthermore, from the results of the hemagglutination-inhibition assays using ferritin and apo-ferritin as inhibitors, we ruled out the direct interaction between ferritin and the lectins used in this study.

When the intact membranes of human erythrocytes were labeled with ferritin-lectins, the number of conjugates bound to the membranes was too small in most cases for the precise assessment of their distribution on the membranes. We have, therefore, labeled the human erythrocyte membranes after treatment with sialidase. It was found that two groups of lectins, which differed from each other in their sugar-binding specificities, gave rise to a different topographic distribution of membrane receptors. Ferritin-conjugated *B. purpurea* and *A. hypogoea* hemagglutinins, both of which primarily recognize the *O*-glycosidically linked sugar chain of human erythrocyte glycoproteins [2], were found in quite a random distribution on the sialidase-treated membranes of human erythrocytes, whereas ferritin-concanavalin A and ferritin-*R. communis* hemagglutinin, both of which bind preferentially to the *N*-glycosidically linked sugar chain of human erythrocyte glycoproteins [1, 3, 16, 17], were found in a clustered distribution. Nicolson [18] has also reported that the aggregation of sialoglycoproteins of the human erythrocyte membrane can be induced with *R. communis* hemagglutinin and concanavalin A. In order to demonstrate this difference between the two groups of lectins more clearly, the number of ferritin particles in small squares with a 250 Å side length (unit area) was counted and the ratio $\sigma(k)/\sigma_{\text{random}}(k)$ as a parameter of clustering was then calculated for squares with a $250 \times k$ Å side length by equation (2) in Materials and Methods. This quantitative treatment further ascertained the different pattern of distribution between two groups of the ferritin-lectins (Fig. 4). Furthermore, to rule out the possibility that this difference between two groups of the ferritin-lectins in their distribution on the cell membrane was not caused by the binding of the lectins, but should be regarded as a simple reflection of the difference in the original distribution of their membrane receptors, the desialized membranes of human erythrocytes were treated with a mixture of concanavalin A and ferritin-*B. purpurea* hemagglutinin. As shown in Figs. 3(f) and 4, the ferritin particles were found in clusters in spite of the fact that ferritin-*B. purpurea* hemagglutinin alone did not show the clustered distribution. This observation indicates that the membrane receptors for *B. purpurea* hemagglutinin are forced to move together with the membrane receptors for concanavalin A, and suggests that the membrane receptors for both lectins possibly reside in the same glycoprotein molecule of the desialized human erythrocyte membrane, and the clustering of the glycoprotein can be effected only when concanavalin A is bound to *N*-glycosidically linked sugar chains of the glycoprotein. However, since Findlay [19] has recently reported that concanavalin A is bound preferentially to Band III glycoprotein of the human erythrocyte membrane, our observation can be interpreted as evidence for the presence of the oligomeric structure containing the major sialoglycoprotein and Band III glycoprotein in the human erythrocyte membrane as suggested by Pinto da Silva and Nicolson [20].

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