Biochimica et Biophysica Acta, 467 (1977) 165—174

BBA 77708

DISTRIBUTION OF THE T-ANTIGEN ON ERYTHROID CELL SURFACES

STUDIES WITH PEANUT AGGLUTININ, AN ANTI-T SPECIFIC LECTIN

EHUD SKUTELSKY [a], REUBEN LOTAN [b], NATHAN SHARON [b] and DAVID DANON [a]

[a] *Section of Biological Ultrastructure and the* [b] *Department of Biophysics, The Weizmann Institute of Science, Rehovoth (Israel)*

(Received November 22nd, 1976)

Summary

The interaction of peanut agglutinin, a lectin with a specificity similar to that of serum T-agglutinin, with human, guinea pig and rabbit young and old red blood cells and bone marrow erythroid cells, both before and after neuraminidase treatment, was investigated. Distribution of the peanut agglutinin receptors was determined by electron microscopical analysis with the ferritin conjugated lectin. In addition, kinetics of agglutination by the lectin were examined with the aid of the Fragiligraph. Rabbit red blood cells of all ages bound peanut agglutinin without neuraminidase treatment. Untreated human and guinea pig red blood cells, as well as immature erythroid cells of guinea pig bone marrow were not agglutinated by the lectin, nor was binding of the lectin to these cells observed by electron microscopy. Treatment with neuraminidase resulted in exposure of peanut agglutinin binding sites on erythroid cells of all stages of maturation and aging. Our findings show that peanut agglutinin receptors are absent from the surface of old circulating red blood cells and extruded erythroid nuclei, in spite of a remarkable in vivo loss of surface sialyl residues. The use of neuraminidase-treated red blood cells, as a model for the study of the clearance of aged red blood cells from circulation seems, therefore, not to be entirely justified.

Introduction

Differentiation, maturation and aging of the mammalian red blood cell are associated with modulations in the density and distribution of surface anionic groups [1—4]. The reduction in negative surface charge that is associated with aging of the circulating red blood cells is due, mainly, to loss of sialic acid residues (*N*-acetylneuraminic acid, AcNeu) [5] which account for at least 95% of the negative charge on the surface of the normal human red blood cell [6]. The

reduction in the density of surface anionic groups, accompanied by the unmasking of cryptic antigenic sites, have been suggested as important factors in the mechanism by which macrophages recognize, phagocytize and destroy senescent red blood cells and extruded erythroid nuclei [7]. Enzymatic removal in vitro of AcNeu residues from red blood cells by neuraminidase resulted in a drastic shortening of the life span of the cells upon reinjection into circulation [8–12] as well as the enhancement of erythrophagocytosis by peritoneal macrophages in vitro [13,14].

Both the in vivo normal modulations of the density and distribution of surface anionic groups and the in vitro enzymatic removal of AcNeu residues from the erythroid cell surface are associated with additional changes of surface properties. Among these are the increase in the number of surface antigenic sites on old and neuraminidase-treated human red blood cells, and on nuclei extruded from rabbit erythroblasts [7], the increase in concanavalin A binding sites on extruded nuclei and neuraminidase-treated rat red blood cells [15], and the decrease in soybean agglutinin binding sites [16] on old human red blood cells.

One of the cryptic antigens which appears after treatment of erythrocytes with neuraminidase is the Thomsen-Friedenreich or T-antigen, which is detected by the naturally occurring plasma panagglutinin termed T-agglutinin, an anti T-antibody present in the plasma of all mammals so far examined [17, 18]. This T-agglutinin is believed to be responsible for the in vivo polyagglutination of red blood cells that occurs in severe bacteremia and viremia, and which is presumably the result of the exposure of the T-antigen by the bacterial and viral neuraminidases.

In the present study we have attempted to find out whether the T-antigen is normally exposed on the surface of senescent red blood cells and extruded erythroid nuclei which have been shown to lose in vivo about 30 and 50% of their surface charge, respectively [1–4]. For this purpose we have employed peanut agglutinin, a lectin specific for terminal non-reducing D-galactose residues, recently purified in our laboratory [19,20]. This lectin was reported to have a specificity similar to that of the anti T-agglutinin [21]. The presence and density of the T-antigen on the surface of human, guinea pig and rabbit red blood cells, as well as on immature erythroid cells and extruded nuclei of the guinea pig bone marrow, both before and after neuraminidase and trypsin treatment, was studied by electron microscopical methods using a peanut agglutinin-ferritin conjugate, The agglutination kinetics of normal, neuraminidase- and trypsin-treated red blood cells by peanut agglutinin and poly-L-lysine was also studied. Our results show that circulating human red blood and guinea pig erythroid cells of all ages and stages of differentiation, as well as extruded nuclei do not bind peanut agglutinin at all, in spite of the physiological reduction in surface sialic acid in some of these cells, and that they bind the lectin only after neuraminidase treatment. Rabbit red blood cells, on the other hand, bind peanut agglutinin without neuraminidase treatment.

Materials

Agglutinin. Peanut agglutinin was purified by affinity chromatography on Sepharose-*N*-ϵ-aminocaproyl-β-D-galactopyranosylamine as previously described [19].

Enzymes. Neuraminidase (*Vibrio cholerae*), free of protease, aldolase and lecithinase-C activities was obtained from Behringwerke AG, Marburg Lahn, G.F.R. Trypsin (bovine) was purchased from Merck AG, Darmstadt, G.F.R.

Chemicals. Ferritin (horse spleen, as 6 times crystallized, cadmium-free) was from Pentax, Kankakee, Illinois; poly-L-lysine, $n = 100$, was from Miles-Yeda, Rehovoth, Israel, and glutaraldehyde from Ladd Research Industries, Burlington, Vermont, U.S.A. "Gravikit", a battery of phthalate ester mixture of predetermined specific gravity for the determination of density and distribution of cells [22] and for differential flotation, was from Miles-Yeda, Rehovoth, Israel.

Methods

Red blood cells and bone marrow cells. Human blood was collected by venipuncture from healthy donors (blood group A, Rh^+) into heparinized test tubes. Density-separated young (top 5%) and old (bottom 5%) red blood cells were obtained by differential flotation [22]. Guinea pig and rabbit blood was collected into heparinized syringes by heart puncture. Red blood cells were separated by centrifugation and aspiration of plasma and buffy coat, washed twice with veronal acetate (0.001 M) buffered saline, pH 7.4, and resuspended in the desired medium. Bone marrow was removed from femurs of anaesthetized adult guinea pigs. The cells were suspended in tissue culture medium M-199 as previously described [2].

Treatment with neuraminidase or trypsin. Suspensions (5% of packed cells, approx. $5 \cdot 10^6$ cells/ml) were incubated in an agitated water bath for 60 min at 37°C with 50 units/ml neuraminidase in a medium containing 0.145 M NaCl, 0.003 M $CaCl_2$ and 0.004 M $NaHCO_3$ pH 6.5 [23]. These are conditions previously used by us and by other laboratories; they are known to remove over 60% of sialyl residues from the surface human red blood cells [9,11]. For treatment with trypsin, the cells at the same concentration as above were incubated for 60 min at 37°C with 0.2 mg/ml of the enzyme in veronal buffered saline. At the end of the incubation, the cells were washed twice with cold veronal buffered saline.

Agglutination assay. Rates of agglutination were automatically recorded in a Fragiligraph (Elmedix Ltd., Tel-Aviv, Israel) [24]. A solution (0.4 ml) containing poly-L-lysine (100 μg/ml) or peanut agglutinin (200 μg/ml) was added to a 2 ml suspension of 1 : 500 packed red blood cells in veronal buffered saline.

Peanut agglutinin-ferritin. The method of Nicolson and Singer for conjugation of lectins to ferritin [25] was generally followed. Peanut agglutinin (150 mg, 1.36 μmol) was dissolved in phosphate buffered saline (pH 6.2, 9 ml) containing D-galactose (1 g), ferritin (850 mg, 1.4 μmol), and ^{125}I-labeled peanut agglutinin (500 μg, 6600 cpm/μg) and the solution was stirred for 30 min at room temperature. 1 ml of 0.5% solution of glutaraldehyde in phosphate buffered saline (pH 6.2) was added dropwise to a final concentration of 0.05%. After gentle stirring for 1 h at room temperature the reaction was stopped by addition of ice-cold glycine (10 ml of 1 M solution in phosphate buffered saline, pH 6.2) and the mixture was dialyzed for 6 h against phosphate buffered saline

(pH 6.2) containing glycine (2 mg/ml). Large aggregates of ferritin were removed by centrifugation at 12 000 × *g* for 15 min and the supernatant was further centrifuged at 160 000 × *g* for 1 h. The pellet was resuspended in phosphate buffered saline, pH 7.4 (20 ml), centrifuged as above and washed again with an additional 20 ml of the same phosphate buffered saline. The pellet was then resuspended in phosphate buffered saline (pH 7.4, 100 ml) and applied at 4°C to a column (2.7 × 17 cm) of Sepharose-*N*-ε-aminocaproyl-β-D-galactopyranosylamine [26]. The column was washed with phosphate buffered saline (pH 7.4) until no ultraviolet-absorbing material was eluted, and then with additional 5 column volumes. The adsorbed peanut agglutinin-ferritin conjugate was eluted with a solution of D-galactose (0.05 M in phosphate buffered saline, pH 7.4), dialyzed extensively against phosphate buffered saline (pH 7.4) and concentrated by ultrafiltration. At each step samples were removed for measurement of ^{125}I in a Packard Gamma Spectrometer to determine the content of peanut agglutinin, and for analysis of total protein [27], using bovine serum albumin as standard. The ferritin content was calculated by subtracting the content of peanut agglutinin from the content of total protein.

The product obtained had a molar ratio of peanut agglutinin to ferritin of 0.96 : 1; its yield in terms of the conjugated proteins was approximately 20%.

Electron microscopy. All cells were labeled after fixation for 15 min at room temperature with the Karnovsky fixative [28], followed by three washes with veronal buffered saline containing 0.1 M glycine. 2 ml of a 5% packed cell suspension was incubated with 1 mg/ml peanut agglutin-ferritin for 30 min at room temperature. In control experiments red blood cells were incubated with 0.2 M D-galactose for 15 min at room temperature and then labeled with the peanut agglutinin-ferritin in the presence of the sugar. The labeled cells were washed three times with veronal buffered saline to remove free, unbound conjugates, fixed for additional 45 min with the Karnovsky fixative and washed again with veronal buffered saline.

The aldehyde-fixed, washed cells in veronal buffered saline were postfixed for 1 h at 4°C with 1% OsO_4 in veronal buffered saline, washed twice in the same buffer and dehydrated in graded ethanol by centrifugation. The cells were resuspended in the desired medium, stained with 50% saturated uranyl acetate in 50% ethanol and embedded in Epon 812 [29]. About 600 Å thick sections (grey interference color) were obtained with an MT-2 Sorval Microtome, mounted on a naked 400-mesh copper grid and coated with carbon. A JEOL-100A electron microscope was used at 80 KV. Counts of ferritin particles were performed on micrographs of perpendicularly sectioned membranes of red blood or bone marrow cells, at a final magnification of × 60 000 as previously described [3]. Twenty different cells of each type from three different experiments were thus counted.

Results

Human red blood cells were not agglutinated by peanut agglutinin, neither before nor after they had been separated into young and old cells (Fig. 1). Similar results were observed with guinea pig red blood cells (Fig. 2). Treatment with trypsin did not affect the lack of agglutination by peanut agglutinin, in

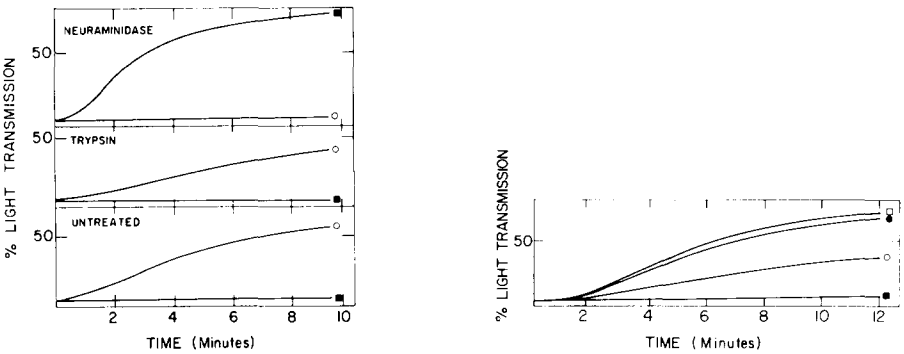

Fig. 1. Rate of agglutination by peanut agglutinin (■) and by poly-L-lysine (○) of neuraminidase-treated, trypsin-treated and untreated human red blood cells.

Fig. 2. Rate of agglutination by peanut agglutinin of rabbit (●) and guinea pig (■) red blood cells, and by poly-L-lysine of rabbit (○) and guinea pig (□) red blood cells.

spite of the considerable reduction in agglutination by poly-L-lysine. However, rapid and strong agglutination was observed after neuraminidase treatment. No agglutination by poly-L-lysine of the neuraminidase-treated cells was observed, indicating that the enzyme removed most of the anionic groups from the cell surface.

In contrast to human and guinea pig red blood cells, those of rabbit were readily agglutinated by peanut agglutinin even without neuraminidase treatment (Fig. 2). The rate of agglutination of the rabbit cells with poly-L-lysine was, however, slow.

Binding of peanut agglutinin-ferritin to human red blood cells. There was no binding of peanut agglutinin-ferritin to normal human circulating red blood cells, neither before nor after they had been fractionated into young and old cells (Fig. 3). Considerable binding of the lectin was observed on neuraminidase-treated human red blood cells (Fig. 4). The ferritin particles attached to the cell surfaces were randomly distributed with an average labeling density of 30 ± 1.5 (S.D.) particles per μm length of membrane. No binding of the lectin was observed on neuraminidase-treated cells that were labeled in the presence of D-galactose (results not shown).

Binding of peanut agglutinin-ferritin to guinea pig erythroid cells. No binding of peanut agglutinin-ferritin was observed on circulating erythrocytes or on suspended erythroid cells of various states of differentiation prepared from guinea

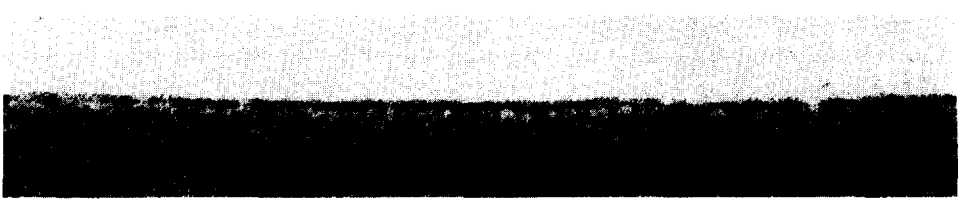

Fig. 3. Old human red blood cell labeled with peanut agglutinin-ferritin. × 100 000.

Fig. 4. Neuraminidase-treated human red blood cell labeled with peanut agglutinin-ferritin. × 100 000.

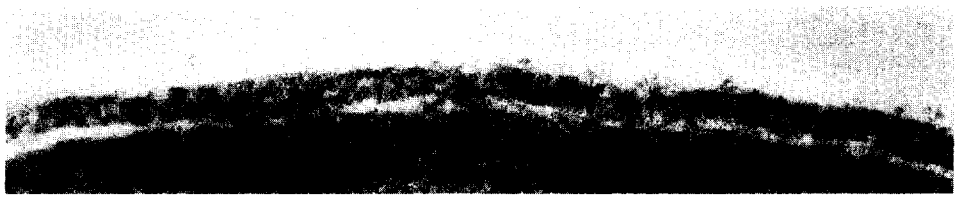

Fig. 5. Extruded erythroid nucleus of guinea pig bone marrow surrounded by narrow cytoplasmic rim and plasmalemma, labeled with peanut agglutinin-ferritin. × 100 000.

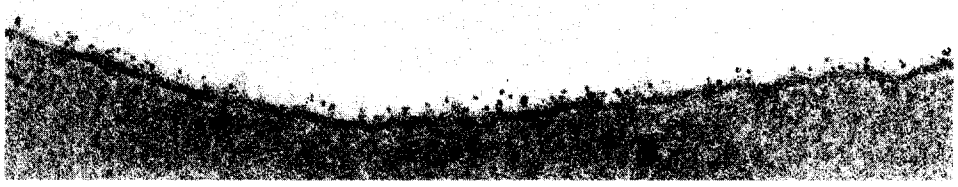

Fig. 6. Reticulocyte from neuraminidase-treated bone marrow of guinea pig, labeled with peanut agglutinin-ferritin. × 100 000.

TABLE I

BINDING OF PEANUT AGGLUTININ-CONJUGATED FERRITIN PARTICLES ON NEURAMINIDASE-TREATED GUINEA PIG ERYTHROID CELLS OF THE BONE MARROW

Number of ferritin particles per micron length of perpendicularly sectioned membranes, counted on micrographs at 60 000 × final magnifications. 20 cells of each cell type in three different experiments were examined.

Cells	Ferritin particles per micron	Standard deviation
Red blood cells *	28.9	5.0
Reticulocytes	27.5	1.9
Late erythroblasts	27.5	2.3
Extruded nuclei	10.2	1.7

* Total population.

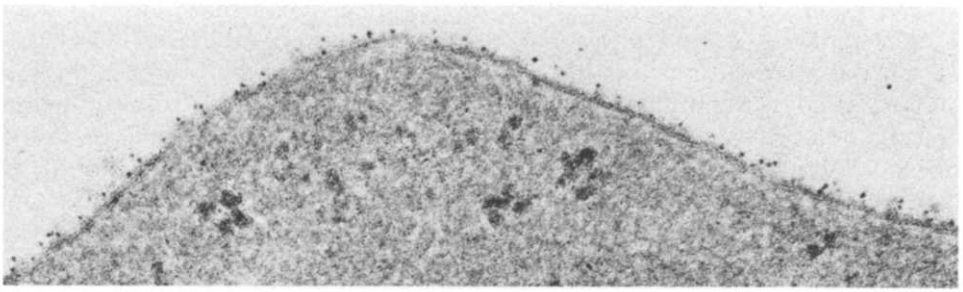

Fig. 7. Orthochromatic erythroblast from neuraminidase-treated bone marrow of guinea pig, labeled with peanut agglutinin-ferritin. × 100 000.

pig bone marrow. Extruded erythroid nuclei as well as late erythroblasts and reticulocytes were also not labeled by peanut agglutinin-ferritin (Fig. 5).

Neuraminidase treatment of red blood and bone marrow cells resulted in the exposure of peanut agglutinin receptors (Figs. 6—8, and Table I). The differences in labeling density between erythrocytes, reticulocytes (Fig. 6) an erythroblasts (Fig. 7) were minor, but the labeling density on extruded nuclei (Fig. 8) was only about one third, as compared to the other erythroid elements (Table I).

Discussion

It is well established that human erythrocytes will not bind peanut agglutinin unless treated by neuraminidase [19,30,31]. The results obtained in the present study clearly show that human senescent cells that contain about 30% less sialic

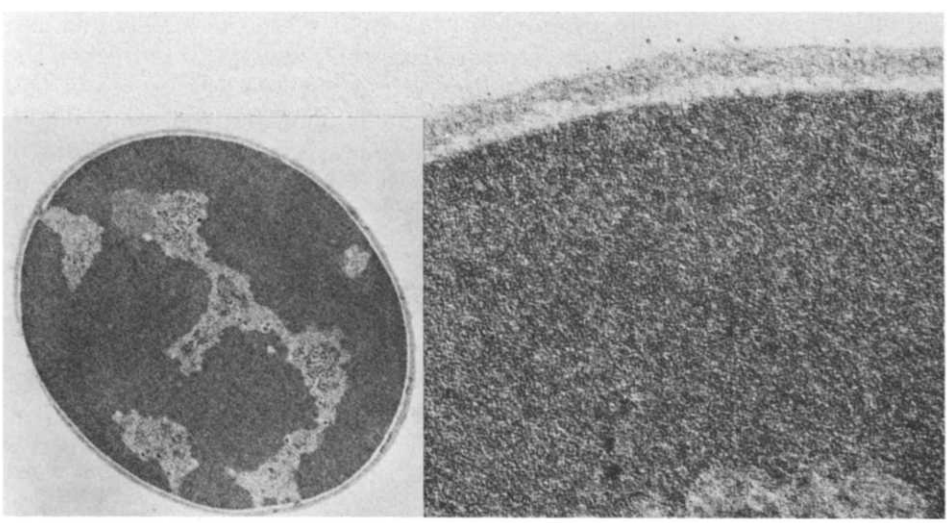

Fig. 8. Extruded erythroid nucleus from neuraminidase-treated bone marrow of guinea pig, labeled with peanut agglutinin-ferritin. × 100 000. Insert: Low power view. × 5000. Note the low labeling density as compared to the other neuraminidase-treated erythroid cells (Figs. 4, 6, 7).

acid than young red blood cells [4,5], or extruded nuclei that have less than 50% of the anionic groups present on newly formed reticulocytes [3,15], are not agglutinated by peanut agglutinin, nor do they bind peanut agglutinin-ferritin. Neuraminidase treatment exposed binding sites for peanut agglutinin on both red blood cells and extruded nuclei, while trypsinization of the cells failed to do so. Sialic acid is known to occupy a non-reducing terminal position in glycoproteins, and is α-ketosidically linked to either D-galactose or to *N*-acetyl-D-galactosamine [32]. It has been suggested that the removal of the terminal sialic acid from red blood cells exposes two types of T-antigens, those with terminal D-galactose and those with terminal *N*-acetyl-D-galactosamine, only the former of which is recognized by peanut agglutinin [18,19].

The T-antigen is believed to be the precursor of blood group MN antigen [33] and indeed desialylated MM or MN substance has been found to be the best inhibitor of the agglutination of neuraminidase-treated red blood cells by both T-agglutinin and peanut agglutinin [18,19].

The lack of exposure of the T-antigen after the removal of sialic acid from red blood cells which have aged in the circulation, as evidenced by our experiments with peanut agglutinin, may be explained by assuming that other glycosidases, such as β-galactosidase, can in vivo cleave the exposed D-galactose residues for which peanut agglutinin and the T-agglutinin are specific. Recent observations by Baxter and Beeley [34] show that in addition to their lower content of sialic acid, old human red blood cells contain less D-galactose than young cells. Indeed, as shown by Marikovsky et al. [16], they contain less binding sites for soybean agglutinin, a lectin specific for both *N*-acetyl-D-galactosamine and D-galactose [35]. The latter finding also indicated that aging of red blood cells in vivo does not unmask *N*-acetyl-D-galactosamine residues.

It has been shown previously that during the expulsion of the erythroid nucleus there is an uneven segregation of anionic groups [2,15], which are selectively excluded from the part of the plasma membrane surrounding the nucleus. This explains why neuraminidase treatment of the plasmalemma surrounding the nucleus failed to reveal more than about a third of the peanut agglutinin receptors as compared with that found for late erythroblasts and reticulocytes. However, the fact that the nucleus is phagocytized immediately after expulsion, and sometimes even before expulsion is terminated [36,37], indicated that the unmasking of T-antigen is not required for phagocytosis to take place.

The finding that receptors for peanut agglutinin are exposed on the surface of rabbit red blood cells may be related to the low surface negative charge [4, 12,38] and sialic acid content [9,12] of these cells, which is less than 50% of that of guinea pig or human red blood cells. The peanut agglutinin receptors of rabbit red blood cells may represent incomplete sialoglycoproteins capable of binding peanut agglutinin but lacking the terminal sialyl residues that mask the corresponding receptors on human and guinea pig red blood cells.

Desialylated erythrocytes are often used for the study of the mechanism of removal of aged erythrocytes from the circulation [8—12,39], as well as the interaction between macrophages and senescent erythrocytes in vitro [13,14]. The results of the present study indicate that the analogy between enhanced clearance from the circulation, or greater erythrophagocytosis of erythrocytes

that have been treated with neuraminidase in vitro, and the recognition and sequestration of aged erythrocytes in vivo, is not entirely justified. Neuraminidase treatment results in the exposure of the T-antigen, a membrane component which is not exposed in the normally aging red blood cells. It seems, however, that the naturally occurring serum anti T-antibody does not usually play a role in the phagocytosis of senescent red blood cells or the extruded nuclei by autologous macrophages as we previously thought [7]. There are indications that serum components are necessary for the recognition of senescent red blood cells by macrophages [14,40]. The nature of antigenic determinant (or determinants) exposed on the membrane of senescent erythrocytes in vivo and its relationship to the clearance of aged red blood cells from circulation, are still to be discovered.

Acknowledgements

The authors wish to thank Mrs. Ora Asher for excellent technical assistance and Mr. Stanley Himmelhoch for photographic work. This study was supported in part by a grant from the United States-Israel Binational Science Fouccation (370) and by a contribution from a friend of the Weizmann Institute in Buenos Aires, Argentina. N.S. is an Established Investigator of the Chief Scientist's Bureau, Israel Ministry of Health.

References

1 Danon, D. and Marikovsky, Y. (1961) C.R. Acad. Sci. 253, 1271—1272
2 Skutelsky, E. and Danon, D. (1969) J. Cell Biol. 43, 8—15
3 Skutelsky, E. and Danon, D. (1970) J. Membrane Biol. 2, 173—179
4 Marikovsky, Y. and Danon, D. (1969) J. Cell Biol. 43, 1—7
5 Cohen, N.S., Ekholm, J.C., Lothra, M.G. and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 229—242
6 Winzler, R.J. (1970) Int. Rev. Cytol. 29, 77—125
7 Skutelsky, E., Marikovsky, Y. and Danon, D. (1974) Europ. J. Immunol. 4, 512—518
8 Hulbhuber, K.-J., Helmke, U. and Geyer, G. (1972) Folia Hematol. Leipzieg 97, 196—203
9 Jancik, J. and Schauer, R. (1974) Hoppe Seyler's Z. Physiol. Chem. Bd. 355, 395—400
10 Gattegno, L., Bladier, D. and Cornillot, P. (1974) Carbohydr. Res. 34, 361—369
11 Jancik, J., Schauer, R. and Streicher, H.J. (1975). Hoppe Seyler's Z. Physiol. Chem. 356, 1329—1331
12 Durocher, J.R., Payne, R.C. and Conrad, M.E. (1975) Blood 45, 11—20
13 Lee, A. (1968) Proc. Soc. Exptl. Biol. 128, 891—894
14 Kay, M. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3521—3525
15 Skutelsky, E. and Farquhar, M.G. (1976) J. Cell Biol. 71, 218—231
16 Marikovsky, Y., Lotan, R., Lis, H., Sharon, N. and Danon, D. (1976) Exptl. Cell Res. 99, 453—456
17 Burnet, F.M. and Anderson, S.G. (1947) Australian J. Exp. Biol. Med. 25, 213—217
18 Uhlenbruck, G., Pardoe, G.I. and Bird, G.W.G. (1969) Z. Immun. Forsch. 138, 423—433
19 Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. (1975) J. Biol. Chem. 250, 8518—8523
20 Pereira, M.E.A., Kabat, E.A., Lotan, R. and Sharon, N. (1976) Carbohydr. Res. 51, 107—118
21 Bird, G.W.G. (1974) Vox Sang 9, 748—749
22 Danon, D. and Marikovsky, Y. (1964) J. Lab. Clin. Med. 64, 668—674
23 Weiss, L., Mayhew, E. and Ulric, K. (1966) Lab. Invest. 15, 1304—1309
24 Danon, D., Marikovsky, Y. and Kohn, A. (1969) Experientia 25, 104—106
25 Nicolson, G.L. and Singer, S.J. (1971) Proc. Natl. Acad. Sci. U.S. 68, 942—945
26 Gordon, J.A., Blumberg, S., Lis, H. and Sharon, N. (1972) FEBS Lett. 24, 193—196
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
28 Karnovsky, M.J. (1965) J. Cell Biol. 27, 137a
29 Luft, J.H. (1961) J. Biophys. Biochem. Cytol. 9, 409—414
30 Irimura, T., Nakajima, M., Hirano, H. and Osawa, T. (1975) Biochim. Biophys. Acta 413, 192—201
31 Carter, W.C. and Sharon, N. (1977) Arch. Biochem. Biophys., in the press

32 Sharon, N., (1975) Complex Carbohydrates, pp. 103—105, Addison Wesley, Reading, Mass.
33 Springer, G.F. and Desai, P.R. (1974) Biochem. Biophys. Res. Commun. 61, 420—425
34 Baxter, A. and Beeley, J.G. (1975) 3rd Int. Symposium on Glycoconjugates, Brighton
35 Lis, H., Sela, B.A., Sachs, L. and Sharon, N. (1970) Biochim. Biophys. Acta 211, 582—585
36 Skutelsky, E. and Danon, D. (1972) Anat. Rec. 173, 123—126
37 Seki, M., Yoneyama, T. and Shirosawa, H. (1965) Acta Pathol. Jap. 15, 295—301
38 Walter, H., Winge, R. and Selby, F.W. (1965) Biochim. Biophys. Acta 109, 293—301
39 Danon, D. (1975) in Physiology and Pathology of Human Aging (Goldman, R. and Rockstein, M eds.), pp. 95—107, Academic Press, New York
40 Stuart, A.E. and Cunning, R.A. (1967) Vox Sang 13, 270—280