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Low Serotonin Uptake by Platelets in Leprosy and a New Approach to Prevent It[1]

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Looking at the long history of leprosy in human civilization, it is surprising that only during the last two decades has the blood of leprosy patients become a subject of intensive investigation. Some major problems related to the clotting system, however, still remain open.

The study of hemostasis in leprosy is significant not only for the treatment of leprosy patients showing coagulation abnormalities and platelet defects ([3]) but also for a better understanding of the mechanisms through which thromboembolic phenomena are less frequent in leprosy ([15]).

Today it is generally accepted that a great many of the coagulation abnormalities in leprosy are due to the presence of a higher level of immunoglobulins, especially of IgM, in the plasma of leprosy patients ([8, 16]). Hypergammaglobulinemia ([4]) can interfere both with the action of coagulation factors as well as with the functions of platelets. However, the fact that not only a quantitative but also a qualitative change occurs in immunoglobulins in leprosy has been neglected for a long time.

Modifications in serum sialic acid levels ([2]) and in platelet membrane sialic acid levels have been observed in leprosy and are almost certainly involved in the abnormal responses of blood constituents in the disease. Sialic acid levels affect a variety of platelet functions such as adhesiveness and aggregation of platelets, release and uptake of serotonin, etc. Serotonin uptake is a particularly relevant parameter not only for blood but also for the nervous system. Indeed, abnormal serotonin uptake observed with platelets can be directly correlated with abnormal functions of tryptaminergic neurons ([7]).

For these reasons, we have investigated changes in mucoproteins in plasma and in platelet homogenates from leprosy patients, considering the sialic acid level to be a measure of structural modifications. We then found a decrease in serotonin uptake by platelets from leprosy patients. Finally, we tried to prevent this decreased serotonin uptake by: a) using normal human plasma instead of plasma from leprosy patients and b) using desoxyfructo-serotonin, a new drug, which has been found to be effective in other cases showing low serotonin uptake. In these experiments desoxyfructo-serotonin was added to the platelet preparation of leprosy patients *in vitro*. It should be noted that the drug has been found to be effective against leprosy in mice after oral administration ([14]).

MATERIALS AND METHODS

Clinical material. This study was conducted on a group of 29 patients with Hansen's disease, hospitalized in the Pavillon de Malte, Hôpital Saint-Louis, Paris, The group was heterogenous in relation to age, sex, and type of leprosy. Diagnosis was based on clinical, bacteriological, immunological, and histological examinations (smears stained by the Ziehl-Neelsen method, whole lepromin and skin biopsies of the Mitsuda test). At the beginning of our experiments, out of these 29 patients, 18 had lepromatous leprosy (LL), five tuberculoid (TT), four borderline leprosy (BL or BT) and two erythema nodosum leprosum

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(ENL), according to the International Classification.

Although these patients had received classical chemotherapy (dapsone, Disulone, Sultirene, rifampin, and Solupred), they were in different clinical states, corresponding to different phases of the disease at the time of the collection of blood for analysis. Some patients were examined repeatedly, in different phases of the evolution of the disease. For this reason the number of examined "cases" (45) is higher than the number of "patients" (29). In all experiments the number of parallel measurements of the same sample was three (Ns = 3). In France the use of thalidomide being not allowed; only one patient had received such treatment before his arrival in France.

We have categorized the patients as follows:

I. *Active phase* (leprosy in evolution)
Clinical reactional episodes in any type, BL (2), BT (10), LL (7); total 19 cases.

II. *Reactive phase* (lepra reaction)
Lepromatous patients with fever, etc., due to incipient ENL but before skin eruptions appear, LL (7), ENL (4); total 11 cases. (sic.)

III. *Stabilized phase* (subsided lepra reaction)
All types of cases, but after stabilization; 15 cases.

During the third phase, we were particularly interested in bacteriologically and immunologically unstabilized severe cases, in which very little, if any, hope exists for recovery. Case 1 (age 36, male) and case 2 (age 20, male) both are bacteriologically positive, untreated lepromatous cases with fever.

The average of cases in Phase I, II, and III, as to age, sex, race, and therapy was nearly identical and very similar to the average of normal cases.

Statistical significance of the results was tested using the Student-Fisher "t" distribution ([6]). The particular methods and denominations used in Table 3 were taken from Graf and Pletscher ([7]).

Biochemical analysis of blood plasma and washed platelets. Blood samples were collected from each patient two or three times during the development of the disease. Twenty-seven healthy donors served as controls. The 27 healthy donors were matched as much as possible to the patients' age, sex, and race. Only 11% of the healthy donors were hospital personnel.

Platelet rich plasma ([5]) (PRP) was obtained by centrifuging citrated blood (1 vol. 3.8% citrate solution plus 9 vol blood) samples at $180 \times g$ for 10 min. The platelet count was made using a Melassez chamber with phase interference optics.

Washed platelets ([1]) were isolated by centrifugation of PRP at $2500 \times g$ at 4°C for 10 min. The sediment was suspended and washed 3 times with 50 vol saline (0.3 M). Washed platelets were homogenized and sonicated.

Protein concentration was determined by Lowry's method, modified by Markwell, *et al.* ([10]), using crystallized bovine albumin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) as a standard.

The mucoprotein fraction of the plasma proteins was precipitated by 5% phosphotungstic acid by the method of Winzler ([18]).

Sialic acid was determined in samples of PRP (0.05 ml), platelet suspension (0.5 ml), and precipitated mucoprotein (0.5 ml) by adding sulfuric acid to a total volume of 1 ml in order to carry out the hydrolysis at a final concentration of 0.05 M H_2SO_4, at 80°C for 30, 45, and 60 min, respectively. Sialic acid was determined with thiobarbituric acid by the method of Warren ([17]), using N-acetyl neuraminic acid (Sigma Chemical Co., St. Louis, Missouri, U.S.A) as an internal standard.

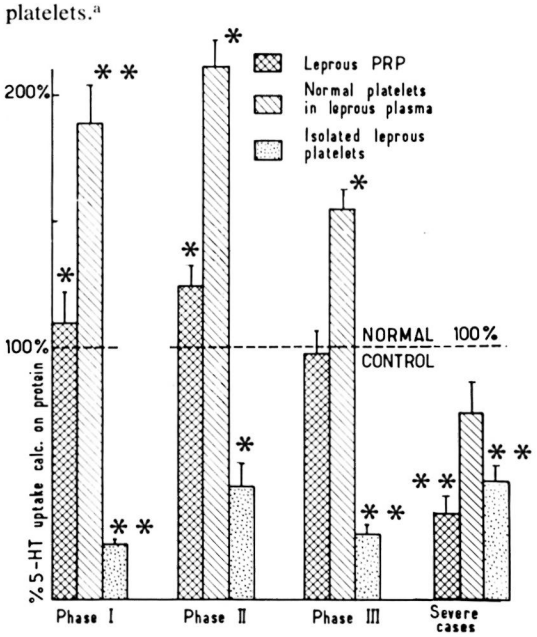
Uptake of 5-hydroxytryptamine-(^{3}H) by platelet rich plasma and by isolated platelets from leprosy patients. Platelet rich plasma (PRP) and the isolated platelet suspension were diluted to a concentration of 10^5 platelets (pls)/mm^3. After 10 min preincubation with shaking at 37°C, 100 μl of PRP or platelet suspension was incubated for 1 min with shaking at 37°C with different concentrations of ^{3}H-labeled 5-hydroxytryptamine (5HT) binoxalate in 0.9% w/v NaCl solution to give final concentrations of 10^{-9}, 5×10^{-9}, 10^{-8}, 5×10^{-8}, and 10^{-7} M, respectively. Two ml of 0.9% NaCl—0.4% EDTA was added to stop the reaction, the platelets were then filtered on nitrated cellulose filter (Millipore, Type GS 0.22 μm),

washed six times with 5 ml of 0.9% NaCl—0.4% EDTA, and the radioactivity on the filter was counted in a liquid scintillation counter (Intertechnic, Plaisir, France) at each concentration. Eight healthy donors served as controls. The percent uptake shown in Fig. 1 represents the average of values obtained at different concentrations and with patients in the three phases of the disease. The same method was used to measure serotonin uptake by normal human platelets in plasma from leprosy patients and vice versa.

In order to study the effect of desoxyfructo-serotonin on the uptake of ^{3}H-labeled 5-hydroxytryptamine by PRP of leprosy patients, 25 μl of 0.9% NaCl (control) and 25 μl of 0.9% NaCl containing desoxyfructo-serotonin, respectively, were added to a constant volume (75μl) of ^{3}H-labeled 5-hydroxytryptamine (5HT) to provide final concentrations of ^{3}H-5HT varying from 10^{-9} to 2.5×10^{-7} M. The final concentration of desoxyfructo-serotonin was 2.15×10^{-7} M in each case. The observed increase in the uptake of ^{3}H-5HT by leprosy PRP is represented in Fig. 3 by the shaded areas (N = 3).

Desoxyfructo-serotonin ([11]) was prepared from the oxalate salt by adding an aqueous solution of $Ca(OH)_2$ and removing the precipitated calcium oxalate by suction. The effect of the compound on platelet shape-change and on the uptake and release of serotonin by normal platelets were measured by the methods reported earlier ([7, 9, 12, 13]).

FIG. 1. Uptake of 5HT by leprous PRP, by normal platelets in leprous plasma and by isolated leprous platelets.[a]



* One asterisk indicates statistically significant change ($p < 0.05$) from the control.

** Two asterisks indicate statistically highly significant change ($p < 0.01$) from the control.

Normal control (N = 8), Phase I (N = 11), Phase II (N = 11), Phase III (N = 9), Severe cases (N = 2).

[a] Average of the uptake of 5HT by leprous PRP, by normal platelets in leprous plasma and by isolated leprous platelets in buffer or in normal human plasma during the three phases of the disease and in severe cases (uptake by normal PRP = 100%).

RESULTS AND DISCUSSION

It has been reported that coagulation abnormalities and platelet defects are due to a high level of mucoproteins, especially IgM ([8, 16]) in the plasma of leprosy patients. The present investigations support the assumption that a structural change related to the sialic acid content both in the plasma and in the platelets is involved in these phenomena.

Mucoprotein level and sialic acid content in the plasma of leprosy patients (Table 1). In addition to a small increase in the total plasma protein level (up to 7%), leprosy patients had marked elevations in plasma mucoprotein concentrations; these increases ranged between 30 and 113% above normal during the evolution of the disease. In spite of a significant increase in the sialic acid concentrations and a significant increase in the percent of sialic acid in the total serum proteins (150% of normal in the third phase of the disease), the sialic acid content of isolated plasma mucoproteins is clearly diminished in the second and third phases of the disease (to 87 and 73% respectively of the normal value). Thus there are definite structural changes in the sugar moiety of the mucoproteins in these leprosy patients.

The sialic acid content of the platelet membrane in leprosy (Table 2). As with the sialic acid content of plasma mucoproteins, the sialic acid content of isolated platelets calculated on platelet protein also diminished.

TABLE 1. *Protein, mucoprotein, and sialic acid concentrations in the plasma of leprosy patients. (Mean ± S.D.).*

Plasma	No.	Proteins mg/ml	Sialic acid mg/ml	% Sialic acid on protein	Mucoprotein mg/ml	% Sialic acid in mucoprotein
Control	27	72.8 ± 5 (100%)	0.64 ± 0.1 (100%)	0.88 ± 0.10 (100%)	0.68 ± 0.10 (100%)	9.48 ± 0.7 (100%)
Phase I (Active)	19	76.4 ± 2.4 (105%)	0.82 ± 0.2[b] (127%)	1.06 ± 0.04[b] (120%)	0.90 ± 0.04[b] (130%)	calculated (95%)
Phase II (Reactive)	11	77.2 ± 4.2[a] (106%)	0.78 ± 0.7[a] (120%)	1.09 ± 0.05[a] (110%)	0.92 ± 0.05[b] (134%)	9.20 ± 0.47[b] (87%)
Phase III (Stabilized)	15	78.0 ± 2.2[a] (107%)	1.03 ± 0.2[b] (161%)	1.32 ± 0.04[b] (150%)	1.50 ± 0.10[b] (213%)	6.90 ± 0.34[b] (73%)

[a] Statistically significant change compared to normal controls, $p < 0.05$.
[b] Statistically significant change compared to normal controls, $p < 0.01$.

The rate of this progressive dimunition is 17%, 19%, and 22% respectively, suggesting a structural change in the carbohydrate moiety of the platelet membrane.

It should be noted that the increase in sialic acid poor protein on the platelet membrane is much higher, 19%, in the third phase than in the two precedent phases where the increase is only 7% and 9%, respectively (Table 2). The change in abnormal protein on the platelet membrane in the third phase is statistically significant ($p < 0.01$). This is probably the main reason that the abnormal serotonin uptake by platelets cannot be corrected by leprosy plasma in the third phase.

As a consequence of the low sialic acid level in the platelets of leprosy patients, a decrease of 20–30% in the platelet aggregation induced by collagen has been observed. This decrease parallels the earlier reported decrease in adhesiveness and aggregability of leprosy platelets ([8]).

TABLE 2. *Sialic acid and protein content in platelet membrane of leprosy patients. (Mean ± S.D.).*

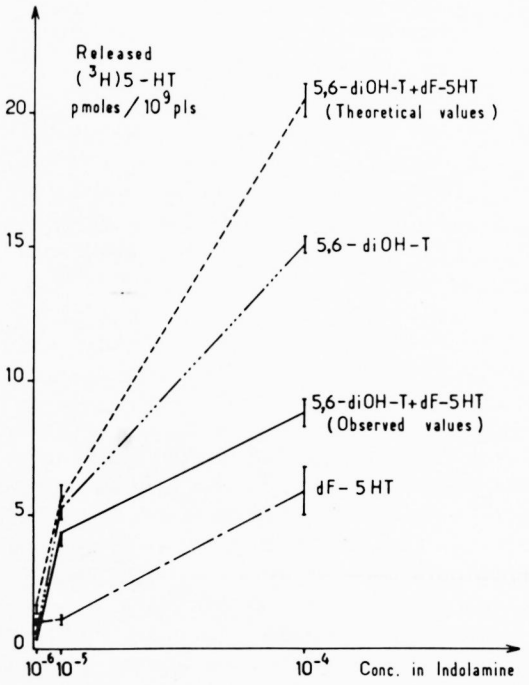
Platelets	No.	Proteins mg/10^9 platelets	Sialic acid μg/mg protein
Control	27	2.51 ± 0.57 (100%)	8.4 ± 0.96 (100%)
Phase I (Active)	19	2.70 ± 0.38 (107%)	7.0 ± 0.38[b] (83%)
Phase II (Reactive)	11	2.74 ± 0.46[a] (109%)	6.9 ± 1.00[b] (81%)
Phase III (Stabilized)	15	3.00 ± 0.43[b] (119%)	6.6 ± 0.74[b] (78%)

[a] Statistically significant change compared to normal controls, $p < 0.05$.
[b] Statistically significant change compared to normal controls, $p < 0.01$.

Uptake of serotonin by leprosy PRP, by normal human platelets in leprosy plasma and by isolated leprosy platelets in buffer or normal human plasma. Isolated platelets of leprosy patients show a significantly diminished uptake of serotonin all through the three phases of the evolution of the disease. The existence of defective platelets is demonstrated by the fact that the low serotonin uptake of leprosy platelets could not be corrected with normal human plasma. In contrast, the sialic acid rich plasma of leprosy patients can prevent the reduced serotonin uptake of the defective leprosy platelets in the first and second phases of the disease and increase by nearly 100% the serotonin uptake in normal human platelets (Fig. 1). In the third phase, the corrective effect of the leprosy plasma is significantly diminished. In this phase the number of severe cases in which defective platelet functions are no longer corrected by their abnormal plasma become more and more frequent. In severe cases, normal human platelets also show a decreased serotonin uptake in the abnormal leprosy plasma. From these experiments the existence in leprosy of both abnormal plasma and defective platelets becomes evident.

Attempt to prevent the low uptake of serotonin by platelets in leprosy. The failure

FIG. 2. Inhibition by desoxyfructo-serotonin of the release of serotonin by human platelets (pls) induced by 5,6-dihydroxytryptamine.



—·····— Release of 5-HT by 5,6-dihydroxytryptamine.

—·—·— Release of 5-HT by desoxyfructo-serotonin.

— — — Theoretical values of the release of 5-HT by a mixture of equimolecular amounts of the two agents.

——— Observed values of the release of 5-HT by a mixture of equimolecular amounts of the two agents, showing inhibition by desoxyfructo-serotonin of the release induced by 5,6-dihydroxytryptamine. Normal human platelets (N = 8).

to prevent the reduced serotonin uptake by platelets in the third phase of the disease in severe cases, using either normal human plasma or leprosy plasma, incited us to look for new approaches to the problem. We have recently reported that some 2-desoxy-2-keto-sugar derivatives of serotonin are activators of the uptake and inhibitors of the release of serotonin by platelets ([9, 13]). We reasoned that these properties could help to correct the reduced serotonin uptake by platelets in leprosy. This is the case for desoxyfructo-serotonin, which exhibits the expected properties. Even at low concentrations ($<10^{-6}$ M), this compound is a powerful inhibitor of the release of serotonin, showing an inhibition of 43.8 ± 6.48% (Mean ± S.D., N = 8) at a concentration of 10^{-4} M. The compound stimulates triptaminergic receptors on the platelet membrane, as demonstrated by the induction of a nearly total (93%) shape-change and of the uptake of serotonin (Table 3).

TABLE 3. *Concentration required to produce 50% of the maximal shape-change of rabbit platelets.*

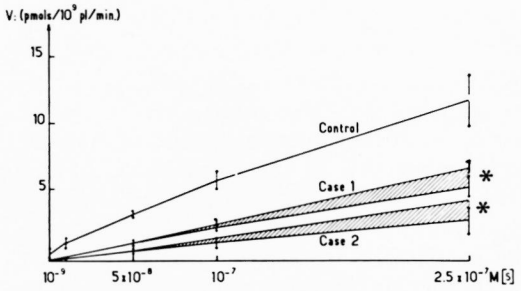
	EC_{50}[a]	SE 95%[b]	Maximal effect
5HT	1.9×10^{-7}	(1.8–2.0)	100%
Desoxyribulo-5HT (oxalate)	8.4×10^{-6}	(4.7–15.0)	74%
Desoxyfructo-5HT	1.7×10^{-5}	(1.5–2.1)	93%
Desoxyfructo-5HT (oxalate)	7.2×10^{-5}	(4.2–12.3)	33%

[a] EC_{50} indicates the concentration inducing half maximal shape-change.
[b] 95% confidence limits.

The release of serotonin induced by 5,6-dihydroxytryptamine is inhibited by desoxyfructo-serotonin, as shown in Fig. 2.

In the case of lepromatous leprosy in the third phase of the evolution of the disease, in severe cases, showing markedly reduced uptake of serotonin in PRP, addition of desoxyfructo-serotonin at a concentration of 2.15×10^{-7} M raised the serotonin uptake significantly (Fig. 3, shaded area).

FIG. 3. Uptake of serotonin by the platelets of leprosy patients before and after addition of desoxyfructo-serotonin.[a]



[S] = Concentration of serotonin.

* The asterisk indicates statistically significant change ($p < 0.05$).

[a] Uptake of serotonin by leprosy platelets at concentrations of serotonin varying from 2.5×10^{-7} M to 10^{-9} M with (upper line) and without (lower line) addition of desoxyfructo-serotonin at concentration of 2.15×10^{-7} M.

The concentration of desoxyfructo-serotonin (2.15×10^{-7} M) was chosen to be half the concentration producing 50% inhibition of the uptake of serotonin by normal platelets in order to avoid an eventual inhibitory effect of the compound on the uptake of serotonin by the leprosy platelets.

CONCLUSION

Measurements of the sialic acid content in isolated plasma mucoproteins and platelets of leprosy patients show a decrease in this sugar component. However, due to the increased mucoprotein concentration, the total sialic acid content of leprosy plasma is increased when compared to normal human plasma. A low sialic acid level was also found with isolated leprosy platelets. Both sialic acid deficient platelets and abnormal plasma seem to be responsible for the abnormal clotting and low incidence of thrombotic phenomena in leprosy patients.

The low content of sialic acid is correlated with a decreased uptake of serotonin by isolated platelets. The change in serotonin uptake by platelets is of particular interest in leprosy because this parameter reflects not only a change in platelet functions but can be directly correlated with defective function of tryptaminergic neurons in the nervous system.

The failure to correct low serotonin uptake in isolated leprosy platelets with normal human plasma during the third phase of the disease is an argument in favor of a definite structural change in leprosy platelets, related to their low sialic acid content.

In the first and second phases of the disease, leprosy plasma can prevent the low uptake of serotonin observed with the isolated platelets. The reactifying effect of leprosy plasma was also demonstrated by its enhancing the serotonin uptake of normal human platelets. In the third phase of the disease, however, leprosy plasma is much less effective in stimulating serotonin uptake in platelets from leprosy patients. The reason for this failure is probably due to the fact that in the third phase, especially in severe cases, not only is the sialic acid level of the protein on the platelet membrane decreased by 22% but also the proportion of this defective protein is increased by 19% (while only 9% in the second phase).

A new approach to prevent the low uptake of serotonin was possible *in vitro* using desoxyfructo-serotonin. The success of this method in severe cases may be of practical interest.

SUMMARY

The plasma of leprosy patients contains high levels of mucoproteins which are deficient in sialic acid. However, due to the increased mucoprotein level, the total sialic acid content of leprous plasma, calculated on protein, is increased when compared with normal human plasma. The low serotonin uptake observed with isolated platelets is probably due to their low sialic acid content. The inability of normal human plasma to correct the diminished serotonin uptake by isolated leprous platelets is in favor of a definite structural change in leprous platelets, related to their low sialic acid content.

In patients with active disease and in those with lepra reactions, leprous plasma itself can correct the diminished uptake of serotonin by the isolated platelets. In patients with subsided lepra reactions, the leprous plasma is much less effective. In severe cases, where serotonin uptake is decreased even in platelet rich plasma, desoxyfructo-serotonin increased the uptake of serotonin.

RESUMEN

El plasma de los pacientes con lepra contiene altos niveles de mucoproteínas que son deficientes en ácido siálico. Sin embargo, debido al incrementado nivel de mucoproteína, el contenido total de ácido siálico en el plasma lepromatoso resulta incrementado cuando se compara con el plasma humano normal. El bajo consumo de serotonina observado con plaquetas aisladas se debe probablemente a su bajo contenido en ácido siálico. La incapacidad del plasma humano normal para corregir el consumo disminuído de serotonina por las plaquetas provinientes de pacientes con lepra esta en favor de un cambio estructural definido en las plaquetas de los pacientes relacionado con su bajo contenido en ácido siálico.

En los pacientes con la enfermedad activa y en aquellos con reacción leprosa, el plasma leproso sí puede corregir el bajo consumo de serotonina por las plaquetas aisladas. En los pacientes con reacción leprosa en regresión el plasma es mucho menos efectivo. En los casos severos donde el consumo de serotonina esta disminuído aún en plasmas ricos en plaquetas, la desoxifructo-serotonina es capaz de incrementar el consumo de serotonina.

RÉSUMÉ

Le plasma des malades de la lèpre contient de grandes quantités de mucoprotéines qui sont déficientes en acide sialique. Néanmoins, par suite de l'augmentation du taux de mucoprotéines, le contenu total en acide sialique du plasma lépreux, lorsqu'il est calculé en quantités de protéines, est accru lorsqu'on le compare au plasma humain normal. La faible incorporation de sérotonine que l' on observe dans les plaquettes isolées est probablement dûe à leur contenu faible en acide sialique. Le fait que le plasma humain normal ne peut rétablir l'incorporation décrue de sérotonine par les plaquettes lépreuses isolées, est en faveur de l'existence de modifications structurales caractéristiques dans les plaquettes lépreuses, modifications qui doivent être en relation avec leur contenu faible en acide sialique.

Chez les malades présentant une affection en évolution, de même que chez ceux qui souffrent de réactions lépreuses, le plasma lui-même peut rétablir l'incorporation diminuée de sérotonine par les plaquettes isolées. Chex les malades qui sont en convalescence de réactions lépreuses, cette activité du plasma est beaucoup moins prononcée. Dans les cas graves, lorsque l'incorporation de sérotonine est diminuée en présence d'un plasma riche en plaquettes, la desoxyfruto-sérotonine augmente l'incorporation de sérotonine.

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