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Increased Sialic Acid in "Blocked" Lymphocytes and Correlation of Spontaneous Release with Clinical State*

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Abstract—*Peripheral lymphocytes from normal subjects and from patients with impaired T cell function were compared with respect to sialic acid. Mean values for total and neuraminidase-susceptible sialic acid were abnormally high in metastatic cancer patients. Spontaneous release of sialic during incubation of lymphocytes in saline was measured for nine metastatic cancer patients and three non-cancers (two anergy, one sarcoidosis). Mean values for both groups were significantly elevated. Correlation between sialic acid release and clinical status was observed in* 11 *out of the* 12 *cases tested.*

Spontaneously released sialic acid was mainly in protein-bound form in normal and cancer cases but the non-cancer group showed high proportions in free and non-protein-bound form. This latter phenomenon was also observed in all groups, normal and diseased, on exposure of the cells to protease 1 *from* A. oryzae.

INTRODUCTION

IMPAIRED T lymphocyte function, as manifested by depression of skin reactivity, lack of mitogen responsiveness, diminished E rosetting etc., is a feature of many disease states including malignancy. Various authors have shown that restoration of T cell activity can occur if the patient's lymphocytes are "washed" [1, 2]. Release of blocking factors into the medium under similar conditions *in vitro* has also been shown [1, 3–5]. The exact nature of these blockers is still uncertain, e.g., whether tumour-specific antigens or antigen–antibody complexes [6, 7] or non-specific entities [2, 8], but they are considered to be sialoglycoproteins with particular affinity for the surfaces of T lymphocytes. It would appear [9] that the presence of sialic acid residues on such molecules may be essential for their immunosuppressive activity. For this reason we decided to analyse peripheral lymphocytes from metastatic cancer patients with blocked T cell function for sialic acid content and the amounts of this released under various *in vitro* conditions. A small group of non-malignant patients with depressed T cell activity were also included and the results for both sets of patients were compared with those for lymphocytes from normal subjects. Previous comparisons of this type have concerned tests of a functional rather than a biochemical nature.

Initially the lymphocytes were incubated in saline for analysis of the total amount of sialic acid spontaneously released into the medium. Incubations were also performed in saline containing protease 1 of *A. oryzae* (brinase) since this agent unblocks cellular immunity both *in vivo* and *in vitro* [10, 11]. In a second type of approach we measured the total cellular sialic acid content and the proportion of this released by incubation with *Vibrio cholerae* neuraminidase. The latter is a measure of most of the sialic acid located on the cell surface [12].

MATERIALS AND METHODS

Blood samples

All subjects were volunteers. The 13 cancer patients had metastatic disease of at least 3 yr duration and were receiving periodic chemotherapy. The five non-cancer patients (three anergy, one sarcoidosis, one toxoplasmosis) were also chronically ill and had shown no response to therapy. All patients, cancer and

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non-cancer, were selected on the basis of a history of negative skin tests for delayed hypersensitivity and depleted E rosetting values, the latter often to levels of only 20–30%. The E rosetting figure for normal subjects is 60–70% [11, 13]. Immediately before our experiments the mean E rosetting for the group of cancer patients was $41 \pm 12\%$ and that for the non-cancer group was $39 \pm 10\%$.

Lymphocyte-enriched blood fractions were collected by IBM cell separator [14] from normal and diseased subjects with the exception of five of the cancer group from whom samples (50 ml) were taken by ordinary phlebotomy. Control experiments showed that lymphocyte sialic acid values were not affected by the method of blood collection used.

Lymphocyte isolation

Isolation commenced within 2 hr of taking samples. Lymphocytes were isolated by centrifugation on Ficoll-Metrizoate [15]. They were suspended in phosphate-buffered saline (Dulbecco A, Oxoid, Ltd.) and washed twice in this medium, followed by centrifugation at 500 ***g*** for 7 min. Platelets were removed by a final slow wash (100 g for 1 min). All preparations comprised 90–95% mononuclear cells.

Cell counts and viability

Counts were carried out in a Burker cytometer under phase-contrast microscopy and concentrations adjusted with phosphate-buffered saline (PBS). Viability, as assessed by trypan blue (0.5 g/l) was greater than 90% both before and after incubation. All experiments were commenced immediately after preparation of the suspensions.

Incubation conditions

All incubations were in a shaking water bath at 37°C for 45 min with PBS alone or containing enzyme. *Vibrio cholerae* neuraminidase (VCN), obtained from Behringwerke AG, Marburg, was used in final concentration of $25\,U/10^7$ cells/ml. A unit (U) is defined as the amount required to release 1 μg of *N*-acetylneuraminic acid from a glycoprotein substrate in 15 min at 37°C. After incubation the cells were removed by centrifugation for 7 min at 500 ***g*** and the supernatants dried down and retained for sialic acid analysis. Protease 1 of *A. oryzae* [16], supplied by Astra AB, Sweden, was used in final concentration of $6\,\mu g/5 \times 10^7$ cells/ml. Supernatants from this treatment, together with PBS controls, were treated with trichloroacetic acid (TCA) to a final concentration of 5% (w/v) and maintained at 4°C overnight. Precipitates were spun down and aliquots of supernatants analysed directly for free sialic acid and, after acid hydrolysis, for bound sialic acid in non-protein form. The latter was calculated by subtraction of free from hydrolysed values. TCA precipitates were acid-hydrolysed for analysis of protein-bound sialic acid. Total cellular sialic acid was determined as previously described [17]. All acid hydrolyses were for 1 hr at 80°C, using 0.1 N HCl. Sialic acid was determined by a modification [17] of the method of Warren [18].

RESULTS

Patients have been allotted numbers for ease of identification. Results for incubation in PBS alone are shown in Table 1. Due to scarcity of material, individual results are single values. The mean total sialic acid loss from normal lymphocytes (eight individuals) was 35 ± 12 nmole/10^9 cells. It is seen that the mean loss from a group of nine cancer patients was 94 ± 66 nmole/10^9 cells, an increase which is significant by "one-tailed" Student's *t*-test ($0.01 < P < 0.02$). The mean value of 128 ± 39 for three non-cancer patients is also significantly greater than normal ($0.01 < P < 0.02$). Examination of the individual cancer patients (Table 1) shows that five cases out of the nine listed gave values which were more than 3 S.D.'s above the normal mean. All these were in an advanced stage of the disease and not responding to treatment. More importantly, cases 3, 7 and 9, all of which showed normal sialic acid release, had at the time of testing responded to treatment and the disease was under control. These findings suggest the possibility that sialic acid release from lymphocytes under these conditions may be a useful indication of disease state. A single exception is found in case 6. This patient's disease was in an active stage at time of testing, but sialic acid release was below the normal mean. Table 1 also shows that each of the three non-cancer patients tested gave abnormally high values. None of these were responding to treatment at time of testing. Taken in general the full results of this Table show that out of a total of nine patients who had not responded to treatment, eight had abnormal values for sialic acid.

Total blood lymphocyte counts for the patients in Table 1 ranged from 684 to

Table 1. Total sialic acid released from lymphocytes during incubation in PBS

Lymphocyte sources	Sialic acid released (nmole/10^9 cells)
(A) Normal subjects	Mean: 35±12 (S.D.)
	(n=8)
(B) Cancer patients	
1. Adenocarcinoma breast	224*
2. Lymphosarcoma (widespread)	108*
3. Squamous cell ca. tongue	30
4. Adenocarcinoma rectum (liver metastases)	94*
5. Anaplastic ca. lung	142*
6. Adenocarcinoma rectum (bone and liver metastases)	27
7. Lymphosarcoma (widespread)	53
8. Undifferentiated ca.	141*
9. Hodgkin's d. (stage 3B)	24
	Mean: 94±66 (S.D.)
	(n=9)
(C) Non-cancer patients	
1. Anergy, depression	146*
2. Anergy, depression	155*
3. Sarcoidosis	82*
	Mean: 128±39 (S.D.)
	(n=3)

*Exceeds normal mean by more than 3 S.D.
S.D = standard deviation.
n = number of individuals.

7330/mm^3 but showed no correlation with sialic acid release.

The total cell content of sialic acid in lymphocytes from seven cancer patients and two non-cancer patients are shown in Table 2 for comparison with normal. The amount of sialic acid released by treatment with neuraminidase (VCN) and its percentage of the total cell content are also given. The normal values are in close agreement with recently published results [19]. The cancer cases as a group had a mean total sialic acid content significantly above normal ($P<0.01$). VCN-susceptible sialic acid was also significantly elevated ($0.01<P<0.02$) in absolute terms but, expressed as percentage of the total, showed no alteration. The two non-cancer subjects tested showed values within normal. Of the cancer group, cases 3, 12 and 13 showed abnormally high values for both total and VCN-susceptible sialic acid and case 7, for which only total was tested, was abnormally high in this respect. Cases 12 and 13 were in an active stage of the disease at the time but cases 3 and 7 had responded to treatment and were in temporary remission. It will be recalled that patient 3 gave a normal value for sialic acid release during PBS incubation (Table 1). The other case in temporary remission, patient 10, showed normal levels according to Table 2. Of the cancer cases with active disease, i.e., 1, 11, 12 and 13, abnormally high values by the parameters in Table 2 were only obtained for cases 12 and 13. Thus, while these results show that metastatic cancer patients as a group had elevated total and VCN-susceptible sialic acid in their lymphocytes, the individual values did not mirror the state of the disease.

Since VCN removes all sterically available surface-located sialic acid [12], subtraction of the VCN-susceptible fraction from the total cellular content should give some measure of the internal sialic acid. By this means it was calculated that the mean value for five cancer patients (117±48) showed significant elevation ($0.02<P<0.05$) over that of lymphocytes from 13 normal subjects, which is 61 ±43. Thus, the anomalies in the lymphocytes

Table 2. Lymphocyte total and VCN-susceptible sialic acid (nmole/10^9 *cells*)

Lymphocyte sources	Total cellular sialic acid	VCN-susceptible moiety	As percentage of total
(A) Normal subjects			
Means ± S.D. ($n=13$)	247 ± 79	178 ± 50	74 ± 10%
(B) Cancer patients			
1. Adenocarcinoma breast	403	n.d.	—
3. Squamous cell ca. tongue	533*	434*	81%
7. Lymphosarcoma (widespread)	484*	n.d.	—
10. Adenocarcinoma breast	247	173	70%
11. Adenocarcinoma ovary	376	255	68%
12. Squamous cell ca. lung	557*	357*	64%
13. Melanoma with multiple metastases	544*	454*	83%
Means ± S.D.	449 ± 102 (7)	334 ± 117 (5)	73 ± 9% (5)
(C) Non-cancer patients			
4. Anergy, depression	296	241	80%
5. Toxoplasmosis	258	164	63%

*Exceeds normal mean by 3 S.D. or more.
S.D. = standard deviation.
n.d. = not determined.

Table 3. Further details of sialic acid components released during incubation in PBS and PBS containing brinase. Sialic acid in nmole/10^9 *cells: mean* ± S.D.

Lymphocyte sources	PBS alone			PBS + Brinase		
	free	non-protein	protein	free	non-protein	protein
Normal subjects (4)*	3 ± 6	13 ± 16	27 ± 6	23 ± 14†	40 ± 8†	23 ± 14
Cancer patients (4)	5 ± 6	11 ± 10	81 ± 82	32 ± 20†	80 ± 38†	55 ± 44
Non-cancer patients (3)	27 ± 13‡	31 ± 27	69 ± 34	56 ± 15	71 ± 52	54 ± 30

*Numbers in parenthesis are No. of patients per group.
† = Significant change due to brinase ($0.02 < P < 0.05$).
‡ = Significant difference from normal PBS value ($0.02 < P < 0.05$).

from cancer patients, taken as a group, appear to concern internally as well as externally located sialic acid.

Nature of sialic acid moieties released by PBS and PBS plus brinase

Aliquots of lymphocytes from four normal subjects were incubated in PBS alone or PBS containing the proteolytic enzyme brinase and the total sialic acid released (cf. Table 1) was differentiated into its free and bound components. Lymphocytes from cancer cases 1, 2, 3 and 4 and non-cancer cases 1, 2 and 3 were similarly tested. Results are given in Table 3. The terms "protein" and "non-protein" in the Table refer to total sialic acid in TCA precipitates after acid hydrolysis and bound sialic acid in TCA supernatants, respectively. Examination of the PBS control values shows that while normal lymphocytes spontaneously released some sialic acid in all three forms,

the major component was protein-bound. A similar pattern emerges for the cancer group, with the increased sialic acid, already described in Table 1 (*ca.* 55 nmole), being attached to protein. PBS controls for the non-cancer cases showed a completely different profile, with the amounts in all three fractions being elevated above normal. All three showed marked increases in free sialic acid and both the anergic patients (cases 1 and 2) gave significant increases in all three parameters.

Effect of brinase

Brinase caused the release of 44 ± 16 extra nmole of total sialic acid from normal lymphocytes and net increases of a similar order in the two diseased groups. As seen in Table 3, normal lymphocytes showed no change in protein-bound sialic acid but there were marked increases in the free and non-protein-bound moieties. Similar patterns were seen with the two groups of diseased subjects except that protein-bound sialic acid was decreased in favour of smaller fractions, presumably due to proteolytic activity taking place in the medium.

DISCUSSION

Sialic acid moieties on cell surfaces and in serum proteins are important in the regulation of many biological properties [12]. T lymphocyte blocking, considered to arise from adherence to the cells of serum sialoprotein, is a feature of advanced cancer and various other disease states. In this connection many authors have measured serum sialic acid levels and attempted to correlate them with the progress of the disease [20, 21]. We considered that analysis of the cells themselves could give a more direct measure of actual lymphocyte suppression. While the ideal approach would be to analyse isolated T cells, observations in this laboratory [22] have shown that the extra manipulations involved in isolation can alter lymphocyte surface sialic acid. Hence, peripheral blood, which consists predominantly of T cells, was used.

The primary aim of this study was to compare blocked lymphocytes from patients with cancer or other diseases with those from normal subjects. Our results show that lymphocytes from cancer patients, as a group, do indeed differ from normal with respect to sialic acid, as demonstrated by significantly elevated mean values for (a) the total cell content of sialic acid, (b) the amount released by VCN and (c) the amount released during incubation in PBS.

The conditions pertaining to the results in Table 1 can be considered analogous to the washing used by other authors to remove blocking factors. An interesting feature of the individual results for the cancer patients in Table 1 was that disease status appeared to be reflected in the amount of sialic acid released. Cases 3, 6, 7 and 9 showed values within the normal range and three of these (3, 7 and 9) were in clinical remission. All others in the group were in active stage of their disease and gave abnormally high values. When it is recalled that all patients had depressed E rosetting and negative skin tests, it appears that the amount of sialic acid spontaneously released can be a sensitive indicator of response to treatment, in contrast to these T cell function tests, whose biological relevance has been questioned [6]. It will be interesting if our findings can be confirmed in patients monitored over a period of treatment.

In contrast, individual values for total and VCN-susceptible sialic acid in lymphocytes from cancer patients (Table 2) showed no general correlation with clinical status. Two of the three cases in clinical remission had elevated values while two of the four cases with active disease gave values within the normal range. If one assumes that the elevation in lymphocyte sialic acid arises either wholly or in part from adherence of blocking compounds, then it is not altogether surprising that the results in Table 2 show a less clear cut pattern than those obtained for spontaneous loss of sialic acid in PBS (Table 1). Blockers are considered to form loosely bound associations with the cell surface [6] and, therefore, are more likely to be selectively detached by gentle procedures such as washing or incubation in saline. The action of VCN, in contrast, is to release virtually all of the surface-located sialic acid. In this context, it is interesting to recall that all lymphocytes tested had been subjected to three washings during preparation, so that any abnormalities detected by PBS incubation could be much more marked *in vivo*.

Five non-cancer patients were included in this study, all of whom were chronically ill and not responding to treatment. The two cases tested for total and VCN-susceptible sialic acid gave normal values, but elevated levels for loss of sialic acid during PBS incubation were obtained in each of the three patients tested by this method. The abnorma-

lities were particularly marked in the two anergic patients. Here, again, therefore, as in the cancer group, high spontaneous loss of lymphocyte sialic acid was associated with poor clinical status.

In cancer or anergy poor response to T cell function tests can be due to low absolute numbers of peripheral T lymphocytes. The elevated values for spontaneous sialic acid release found in the present study are, however, unlikely to be related to a preponderance of B or null cells. Our own unpublished observations have shown that T lymphocytes in peripheral blood have approximately three times the sialic acid content of the non-T cell population and the electrophoretic studies of other authors [23, 24] indicate that T cells have higher surface sialic acid than B cells. The results are also unlikely to be due to abnormally high numbers of T cells in the samples. In patients with advanced cancer [1, 11] or anergy (Thornes, unpublished observations) enumeration of T cells by E rosetting after unblocking indicates that absolute T cell numbers in these patients are no greater than normal. The possibility of increased granulocyte contamination in some samples from advanced cancer patients, as found by Currie *et al.*, [25] cannot, however, be ruled out as an influence on our results and is at present being investigated.

Holland *et al.* [11] showed that exposure to brinase *in vitro* restored to normal the depressed E rosetting of lymphocytes from cancer patients. Subsequently [1] it was found that a similar effect could be obtained by merely washing the lymphocytes. Our own experiments have shown that exposure to saline alone released sialic acid from normal lymphocytes and significantly more from the two diseased groups. The release was mainly in the form of protein where normal and cancer patients were concerned, but high proportions of free and non-protein bound sialic acid were noted in the non-cancer patients. The reason for this is not clear but it may possibly involve release of lysosomal enzymes.

The additional presence of brinase, while causing release of extra sialic acid from all three groups of lymphocytes, did not significantly distinguish the normal from either of the diseased groups. In all three groups, large proportions of the brinase-released sialic acid were in the free form. This suggested the possibility that the purified enzyme preparation might be contaminated with neuraminidase. However, no such activity could be detected on incubation of brinase with the synthetic substrate NANA-lactose. It is possible that the production of free sialic acid during incubation of lymphocytes with brinase may be secondary to an effect on lysosomal permeability. Brinase increases lysosomal permeability in intact ascites tumour cells [26].

In conclusion, the present results indicate that in cancer patients the amount of spontaneously releasable sialic acid from lymphocytes is more closely related to clinical status than is the E rosetting value. The release is not due to cell death during incubation. It is not possible at present to state whether the extra sialic acid derives from serum glycoprotein adhering to the lymphocyte surface; while this may be so, our finding that estimated internal sialic acid levels were significantly raised suggests that synthesis and secretion of sialo-complexes by the lymphocytes [7] may be involved. Further work will be needed to examine this possibility, as well as to monitor spontaneous release in individual patients before and during treatment in conjunction with serum sialic acid levels. The important feature of the present findings is the proposal that measurement of spontaneous release may serve as a reproducible biochemical monitor of disease state and response to treatment.

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