PRELIMINARY COMMUNICATIONS

Lymphocytotoxins in Leukaemia

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Summary

Fifteen out of 51 patients with leukaemia have produced a lymphocytotoxin in their serum which reacts with their own and a high percentage of all other lymphocytes. This is probably a protein, but not an antibody. It has been detected only before or early during cytotoxic treatment, or during a relapse. It is postulated that the lymphocytotoxin is a specific protein produced by the leukaemic cells, which may facilitate their spread in the body.

Introduction

During a study of the immune changes of leukaemia patients two factors have been found in their serum. The first is an autoantibody similar to that discovered by Doré *et al.* (1967) and Mathé (1970), and the second is a lymphocytotoxin. The lymphocytotoxin was discovered by using the standard cytotoxicity test designed by Terasaki and McClelland (1964) for tissue typing. It gave the cells that it killed an unusual appearance when compared with those cells killed by an antibody under the conditions of this test. In this paper the occurrence of the lymphocytotoxin and the results of the various tests that were carried out to determine its properties are discussed.

Methods

The cytotoxicity test (Terasaki and McClelland, 1964) was used as the basis of all the investigations, but variations, such as the omission of complement and alterations of the temperature and duration of the test, were introduced to see whether they reduced or increased the numbers of cells killed by the lymphocytotoxin. As usual all sera which killed more than 20% of the lymphocytes were regarded as positive. HL-A antisera obtained from the N.I.H. at Bethesda, Maryland, were used as controls. Thirty antisera covering HL-A antigens 1, 2, 3, 5, 7, 8, 9, 10, 11, 12, and 13 were subjected to the same tests as the lymphocytotoxins, and comparisons were made between the activity of both test and control sera. Sera from 31 normal individuals and 74 patients with other diseases were screened for lymphocytotoxins. These included patients with other forms of cancer, other haematological diseases, and unrelated diseases such as glandular fever and rubella. Three of these patients were found to have a lymphocytotoxin in their serum, two were patients with a neutropenia, and the third had myeloid metaplasia. All sera including the negative ones were screened against lymphocytes from at least 100 donors.

Trypsin digestion was carried out by a modification of a standard method (Huehns, 1968); 0.25 ml of serum was incubated with 0.35 mg of trypsin in 1 ml of 1/1,000 N HCl at 37°C for four hours at pH 7.0. In the original method the incubation is carried out at pH 9.0 for two hours, but in the present experiment it was impossible to raise the pH to 9.0 as it would have killed the lymphocytes. The lower pH of this experiment slows the rate of digestion so the incubation time was doubled.

In the ribonuclease digestion experiment 0.025 ml of 1% aqueous solution of ribonuclease was added to 1 ml of serum. This mixture was incubated at 37°C for four hours.

Molecular size was investigated by dialysing aliquots of serum in $\frac{1}{4}$ in (6.3 mm) dialysis tubing against phosphate-buffered saline at pH 7.3 for 24 hours. One would expect that all substances with a molecular weight of less than about 10,000 would be dialysed out by this method (Craig *et al.*, 1969).

Results

OCCURRENCE OF LYMPHOCYTOTOXIN

Fifty-one patients with leukaemia were tested, 25 before or during the first two weeks of treatment, and the lymphocytotoxin was detected in 11. A further eight were tested only in relapse, three of whom had a lymphocytotoxin. As Table I shows, the lymphocytotoxin was found predominantly in patients with

TABLE I—Distribution of Lymphocytotoxin in Relation to Type of Leukaemia

Type of Leukaemia	Before Two Weeks' Treatment		During Relapse	
	Total Tested	No. Lymphocytotoxin Positive	Total Tested	No. Lymphocytotoxin Positive
Acute myeloid ..	10	6	5	3
Chronic myeloid ..	6	4	0	0
Acute lymphatic ..	7	0	2	0
Chronic lymphatic ..	2	1	1	0
Total	25	11	8	3

Eighteen patients were tested only in remission. One patient with chronic lymphatic leukaemia had a detectable lymphocytotoxin during remission.

myeloid leukaemias. Of the 15 patients with acute myeloid leukaemia tested nine had a lymphocytotoxin, as did four of the six patients with chronic myeloid leukaemia. The other two leukaemia patients with the lymphocytotoxin had chronic lymphatic leukaemia, and in one of these it was found during remission. Although nine patients with acute lymphatic leukaemia were investigated, seven within the first few days of treatment, no lymphocytotoxins were detected in any of them. The remaining 18 patients were tested only during remission.

As cytotoxic treatment proceeded the activity of the lymphocytotoxin was no longer detected (Table II). In two patients, Cases 12 and 13, from whom the first two samples were obtained within a week of each other, the second and post-treatment sample had a weaker activity than the first. In five patients the lymphocytotoxin was detected during relapse. Two of these patients had had a lymphocytotoxin in their serum in the first few days of treatment and two were not tested before their relapse. The fifth patient, although tested early during his disease, had a detectable lymphocytotoxin only during relapse.

All the lymphocytotoxic sera were tested against the patients' own lymphocytes and those from at least 100 donors. In each case it reacted positively with their own lymphocytes and with a

TABLE II—Occurrence of Lymphocytotoxin in Relation to Treatment

Case No.	Type of Leukaemia	Before Treatment	1st Week	2nd Week	Samples During Remission or Partial Remission	Relapse	After Further Treatment
1	Acute myeloid	+	+	—	1 negative in 2 months 7 negative in 6 months		
2			+				
3			+				
4			—	—		+	—
5			+	+	13 negative in 14 months 3 negative in 5 months	+	
6	Subacute myeloid	+		—		+	
7				—		+	
8	Chronic myeloid	+		+	7 negative in 16 months 8 negative in 12 months		—
9				+	3 negative in 11 months	+	
10				+	4 negative in 4 months		
11	Chronic lymphatic	+	+				
12			+				
13			+		2 negative in 6 months		
14			+		+		

+ = Lymphocytotoxin detected. — = Lymphocytotoxin not detected. Blank = Not tested.

TABLE III—Tests for Characterization of Lymphocytotoxin. Results given as Percentage of Cells Killed

Case No.	Complement Dependence		Antihuman IgG Inhibition		Antihuman IgM Inhibition		Temperature Difference		Time Difference	
	+C	—C	+	—	+	—	4°C	37°C	5 Min	60 Min
1	30	50	18	47	45	47	20	20	>95	>95
2	33	56	50	48	50	48	38	29	>95	>95
3	32	36	50	50	50	50	28	25	>95	>95
4	31	22							>95	>95
5			33	25	26	25			>95	>95
6	>95	>95					80	>95	>95	>95
7	24	21					23	33	>95	>95
8	82	75	>95	>95	>95	>95	13	25	>95	>95
9	20	23	34	28	22	28	30	25	>95	>95
10			50	<10	<10	<10	30	25	>95	>95
11			50	50	75	50	20	10	>95	>95
13	23	29					75	60	>95	>95
Mean difference ± S.D.	+ 4.1 ± 11.05		— 3.37 ± 18.7		— 2.5 ± 9.8		+ 3.89 ± 9.8		— 1.64 ± 4.7	

high percentage of the other lymphocytes—for example, serum from Case 6 killed lymphocytes from 96% of the donors, while that from Case 13 reacted with 82% of the lymphocytes.

The strength of the lymphocytotoxin was measured by comparing the percentage of lymphocytes killed in each reaction with the donors' cells. This varied greatly between the different sera—for example, Case 6 gave persistently high kills, frequently of over 95%, while Case 5 gave very low kills and sometimes negative results. The serum obtained from any one patient at a given time gives consistent results when tested against a panel of lymphocytes though, as already mentioned, samples collected from the same patient at different stages of their disease gave very different results.

CHARACTERIZATION OF LYMPHOCYTOTOXIN

Complement Dependence.—Two plates containing nine lymphocytotoxic sera and two control plates were incubated at 37°C for one hour with lymphocytes from the same donor. Halfway through the incubation complement was added to one plate of both lymphocytotoxic sera and HL-A antisera. At the end of the hour all four plates were read microscopically. Whereas both plates of lymphocytotoxic sera gave positive results, whether or not complement had been added (Table III), only the control plate with complement was positive. On this plate the donor typed HL-A1, HL-A2, and HL-A8.

IgG and IgM Inhibitions.—Plates containing eight lymphocytotoxic sera and control plates were incubated with either antihuman IgG or antihuman IgM. These sera were subsequently used in the cytotoxicity test together with an unreacted plate of test and control sera. Neither antibody decreased the number of cells killed by the lymphocytotoxin, though both inhibited the activity of the HL-A antisera (Table III).

Temperature Dependence.—Antibodies are normally dependent on a narrow range of temperatures for their activity. In the case of the HL-A antibodies used their optimum temperature was 37°C. The lymphocytotoxin reacted equally strongly, however, at 4°C, room temperature, and 37°C (Table III).

Incubation Time.—This had little effect on the lymphocytotoxin's activity. Twelve of the sera were incubated for lengths of time varying between five minutes and two hours, and even after five minutes the percentage of dead cells was high (Table III). An hour's incubation was taken as the standard for comparison.

pH Variations.—Lymphocytes are very sensitive to changes in pH and die if this varies much from 7.3. As a result it proved impossible to evaluate the effect of pH variations in the lymphocytotoxin's activity.

Molecular Size.—The only tests that have been carried out so far to determine the molecular size of the lymphocytotoxin are the dialysis experiments. When the lymphocytotoxic sera that had been dialysed were used in the cytotoxicity test their activity was still present, which suggests that it has a molecular weight of greater than 10,000 (Table IV). As a control normal group AB serum was dialysed and negative results were obtained.

TABLE IV—Results of Dialysis Experiments expressed as Percentage of Cell Killed

Cells	Serum from Case 7		Serum from Case 8	
	After Dialysis (%)	Undialysed (%)	After Dialysis (%)	Undialysed (%)
1	21	19	33	36
2	33	33	26	49
3	35	30	41	43
4	21	33	30	29

Trypsin Digestion.—The lymphocytotoxic sera that had been digested by trypsin had lost their activity when used in the cytotoxicity test subsequently. As controls neat lymphocytotoxic serum and lymphocytotoxic serum plus 1 ml of 1/1,000 N HCl were incubated at 37°C for four hours, and the activity remained in the sera. Normal serum was subjected to all these tests and remained unaffected (see Chart). As trypsin digested the lymphocytotoxin it is probably a protein.

Effect of trypsin digestion on percentage of cells killed.

Ribonuclease Digestion.—The activity was still present in the lymphocytotoxic serum after ribonuclease digestion, showing that it is not ribonucleic acid (Table V).

TABLE V—Ribonuclease Digestion expressed as Percentage of Cells killed

Cells:	1	2	3	4
Lymphocytotoxin*	38	39	42	43
Lymphocytotoxin + ribonuclease	45	37	34	37
Normal serum	<10	<5	<5	<5
Normal serum + ribonuclease	<5	<5	<5	<5

*Serum from Case 8.

Discussion

The lymphocytotoxin reacts equally strongly with or without complement, its activity is not inhibited by either antihuman IgG or antihuman IgM, and, furthermore, it reacts rapidly and over a wide range of temperatures. These findings are not those of normal antibody activity, indicating that the lymphocytotoxin is not an antibody. The finding that the lymphocytotoxin's activity is not dialysable but is destroyed by digestion with trypsin suggests that it is a protein. There is evidence from comparisons of platelet-free and non-platelet-free lymphocyte

suspensions that suggests that the lymphocytotoxin may also react with platelets, but as so far no work has been done with other cells it is impossible to say whether it reacts with a wider range of cells than that suggested by the name lymphocytotoxin.

The lymphocytotoxin is found predominantly in patients with myeloid leukaemia mainly before or during the first two weeks of treatment. After the beginning of treatment the lymphocytotoxin's activity becomes weaker and finally undetectable. This may be because either it is destroyed by cytotoxic drugs or these drugs are destroying the cells that are producing it. In a few patients it has been detected during relapse despite their receiving these drugs.

The problem of what produces the lymphocytotoxin remains. One explanation is that it may be produced by the host in an attempt to eliminate the malignant cells. As it kills normal lymphocytes, including those of the host, this seems unlikely. Moreover one would expect that such a defence mechanism would involve the production of antibodies and, in fact, weak autoantibodies have been found in some patients with leukaemia by Doré *et al.* (1967), Mathé (1970), and me. If it were a product of the host it would probably be present at all times, whereas this substance is not detectable when the patient is in remission or partial remission.

Another possibility is that it is produced by the leukaemic cells. If this is the case it may be formed in several ways. It may be either a non-specific breakdown product of these cells or a specific antilymphocyte substance. The finding that it is not detectable when the largest number of abnormal cells are being killed—namely, when the patient is receiving cytotoxic treatment—makes it unlikely that the lymphocytotoxin is a breakdown product of leukaemic cells. The findings presented are consistent with the suggestion that the lymphocytotoxin is a specific substance that kills lymphocytes and in this way might inhibit the normal response of the body to foreign cells. This would facilitate the spread of the malignant cells and may be one of the causes of the rapid progress of some leukaemias. It would also explain why it has never been detected in acute lymphatic leukaemia patients, for if they produced a lymphocytotoxin they would be hindering the spread of the cells, not enhancing it. Against this explanation is the failure to detect the lymphocytotoxin whenever there are leukaemic cells present, as when the patient is in incomplete remission. Possibly the lymphocytotoxin is mainly released at the site of production of the cells and is detectable in the peripheral blood only when there is a high turnover of leukaemic cells. Thus it is found in the peripheral blood only before treatment, or during relapse, when there are large numbers of abnormal cells present.

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