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Immunosuppressive Effect of a Human Hepatic Glycoferroprotein, α2H Globulin

A STUDY ON THE TRANSFORMATION OF NORMAL HUMAN LYMPHOCYTES

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Summary. A macroglycoferroprotein of hepatic origin, α2H globulin, the serum level of which increases a few weeks or months before local recurrence of metastases, has been essayed for its immunosuppressive activity. The study was carried out using the lymphoblastic transformation test and was judged by tritiated thymidine incorporation and microscopic examination.

PHA-induced blast transformation of 97 per cent of normal donor lymphocytes is inhibited by 100 μg/ml of α2H globulin. This inhibitory effect is proportional to the quantity of added α2H globulin. It is obvious with a concentration of 2–5 μg/ml, a frequently observed level in the serum of patient with tumours. Preincubation of lymphocytes with α2H globulin renders more effective the inhibitory action on PHA-induced transformation.

A mechanism of competition between PHA and α2H globulin is suggested by preincubation and the inhibitory effect related to the doses. However, microscopic observation shows that α2H globulin acts on the earliest events occurring to the stimulated lymphocytes, by inhibiting cytoplasmic RNA and protein synthesis. The α2H globulin effect may not only have an immunosuppressive activity but it may have a more general effect, for example blocking or modifying cellular respiration.

INTRODUCTION

In previous work (Buffe, Rimbaut and Burtin, 1968, 1973; Buffe, Rimbaut, Lemerle, Schweisguth and Burtin, 1970; Rimbaut, 1973) we showed the presence of a glycoferroprotein, α2H globulin, in the serum of patients with serious diseases, especially those with malignant diseases. When we followed the variation in the levels of α2H globulin we noted that the level increased about 1 or 2 months before local recurrence or the clinical appearance of metastases. This observation has been confirmed by Martin, Charlionnet and Ropartz (1971), Wada, Anzai, Takahashi and Sakamoto (1970) and Tatarinov (personal communication).

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What is the role of α2H globulin in the growth or the spread of tumours? Does purified α2H globulin exert an immunosuppressive effect?

Since the work of Kamrin in 1959, a number of reports (Mowbray, 1963; Mannick and Schmid, 1967; Cooperband, Bondevick, Schmid and Mannik, 1968; Riggio, Parillo, Bull, Schwartz, Stenzel and Rubin, 1971) have indicated that α2 globulin-rich fractions of mammalian sera are inhibitors of the immune response *in vivo* and *in vitro*. Possibly α2H globulin is one of these α2 globulins. To verify this hypothesis we have tested *in vitro* the reactivity of human lymphocytes in the presence of different levels of α2H globulin.

Because PHA-induced lymphoblastic transformation is regarded as a good indication of lymphocyte reactivity, we have used this method to test our α2 hepatic globulin (α2H).

MATERIALS AND METHODS

α2H globulin

This was extracted, isolated, and purified from human livers with tumours according to the technique described in a previous paper (Buffe, Rimbaut, Fuccaro and Burtin, 1972) and used at different concentrations ranging from 2 μg/ml to 1 mg/ml.

Lymphocytes cultures

Purified human lymphocytes from forty-three healthy donors and three haemochromatosis patients were prepared by the method described by Harris and Ukaejiofo (1969). The lymphocytes from heparinized human blood were isolated by sedimentation in Ficoll–Triosil mixed medium.

After washing with culture medium 199 (Difco TC 199) the lymphocytes were cultured in the same medium containing 20 per cent inactivated human AB serum.

The cultures were established with $1{\cdot}5 \times 10^6$ cells in 2 ml of medium and kept in an incubator at 37° for 5 days.

α2H globulin was added to the medium at the beginning of the culture at a final concentration of 2–1000 μg/ml.

Phytohaemagglutinin (Difco M)

0·1 ml was added either at the same time as the α2H globulin, i.e. at the start of the culture, or 24 hours later.

Triplicate or quadruplicate cultures were determined for each experiment.

Synthesis of DNA

This was determined in the cultures by the incorporation of tritiated thymidine (1 μCi for 1 ml of medium) added to the culture tubes on day 5.

On day 6, 0·5 ml of lymphocyte suspension was pipetted out to study the morphology and the viability of the cells.

The incorporation of [^{3}H]thymidine in DNA was determined in the remaining cells by counting the radioactivity in a Packard scintillation counter. The data presented is the mean of each experiment.

RESULTS

I. STUDY OF LYMPHOBLASTIC TRANSFORMATION AS JUDGED BY INCORPORATION OF TRITIATED THYMIDINE IN LYMPHOBLASTIC DNA

The experiments showed that 100 μg/ml of α2H globulin exerted an inhibitory effect on DNA synthesis induced by PHA (Table 1).

TABLE 1

In vitro INCORPORATION OF [^{3}H]THYMIDINE IN HUMAN LYMPHOCYTES IN THE PRESENCE OF PHA AND 100 μg/ml OF α2H GLOBULIN

Case number	Spontaneous transformation (ct/minute)	PHA-induced transformation (ct/minute)	α2H-induced transformation (ct/minute)	PHA-+α2H-induced transformation (ct/minute)	Suppressive index (I_s) $\left(\frac{\text{ct/min PHA}+\alpha2H}{\text{ct/min PHA}}\right)$
1	1923	112,027	3746	29,043	0·25
2	511	35,421	973	4237	0·12
3	493	41,328	507	9761	0·23
4	725	61,517	5375	29,122	0·47
5	898	112,414	749	7394	0·07
6	913	121,076	4731	41,319	0·34
7	580	46,084	602	3431	0·07
8	327	24,932	979	5945	0·24
9	2340	125,969	1509	58,709	0·47
10	613	9139	895	2907	0·32
11	1461	8467	3372	3211	0·39
12	1189	157,013	10,162	63,976	0·40
13	473	61,127	1237	7701	0·12
14	1192	97,345	1471	20,519	0·20
15	531	60,052	427	8944	0·10
16	1826	127,005	30,316	7894	0·14
17	903	48,224	1979	21,530	0·40
18	2061	123,826	9172	9335	0·07
19	1296	60,413	25,089	12,056	0·19
20	1784	121,682	1819	68,573	0·56
21	1007	53,589	592	23,221	0·40
22	1826	120,106	2023	37,005	0·35
23	458	47,604	864	19,211	0·40
24	948	48,224	931	14,222	0·29
25	1501	113,133	3709	73,656	0·64
26	1219	71,437	4436	18,708	0·26
27	478	5177	501	1247	0·24
28	503	5546	419	1869	0·33
29	619	57,479	831	31,843	0·55
30	356	37,283	1052	20,501	0·54
31	252	33,395	1103	14,894	0·40
32	2137	91,000	3010	67,086	0·73
33	534	51,005	7931	19,304	0·37
34	1155	65,980	1616	31,999	0·40
35	1754	132,295	2597	17,327	0·13
36	376	38,326	1732	6483	0·17
37	607	47,340	1001	19,732	0·40
38	1071	58,954	1533	21,958	0·30
39	963	67,300	994	35,007	0·52
40	1268	82,401	1014	43,038	0·52
41	485	112,917	20,543	12,056	0·10
42	739	61,203	29,433	10,122	0·16
43	1027	59,453	33,201	11,729	0·19
44*	9380	21,897	10,007	18,626	0·85
45*	386	1129	627	1109	0·98
46*	13,971	28,177	11,181	21,276	0·75

* Haemochromatosis.

To facilitate the reading of the results we determined a suppressive index (I_s). I_s = [(ct/minute in the presence of PHA and α2H)/(ct/minute in the presence of PHA alone)].

We considered that there was an inhibitory effect if the index was below 0·7; when I_s was in the range of 0·7–1, α2H globulin was considered to have no effect and when I_s was greater than 1, α2H was considered to stimulate the lymphocyte response. These data are summarized in Table 2.

TABLE 2

REFERENCE VALUE OF SUPPRESSIVE INDEX (I_s) IN THE PRESENCE OF α2H

$I_s < 0{\cdot}7$	Suppressive effect
$0{\cdot}7 < I_s < 1$	No effect
$I_s > 1$	Stimulating effect

In forty-two out of forty-three healthy donors (97 per cent) α2H globulin at a dose of 100 μg/ml exerted a suppressive effect.

In one normal case and in the three cases of haemochromatosis the suppressive index was not significantly altered (0·75, 0·79, 0·85 and 0·98 respectively) (Table 3).

TABLE 3

NUMBER OF CASES ACCORDING TO IMMUNOSUPPRESSIVE INDEX IN THE PRESENCE OF PHA + α2H GLOBULIN (100 μg/ml)

$I_s < 0{\cdot}25$	18 cases	(97 per cent normal donors)
$0{\cdot}25 < I_s < 0{\cdot}70$	24 cases	
$I_s > 0{\cdot}70$	4 cases*	
	46 cases	

* Three cases of haemochromatosis.

The suppressive effect is proportional to the quantity of added α2H globulin.

Five cases (numbers 18, 19, 41, 42 and 43) were studied with different doses ranging from 2–1000 μg/ml of purified α2H globulin. The suppressive index was found to lie between 0·02 and 0·75 (Table 4).

The effect of preincubation with α2H globulin on PHA-stimulated lymphocytes was also studied. In four cases the lymphocytes were preincubated with the purified globulin at the same doses (from 2–1000 μg/ml) before adding PHA, without washing the cells; hence α2H globulin was in the medium till the end of the culture.

Since we tested the lymphocytes of only four donors under these conditions, each experiment was made in quadruplicate and a mean value taken. The results in one case (number 42) are summarized in Table 5.

In all cases after preincubation the inhibitory action was greater; the suppressive index was below that found when the α2H was added at the same time as PHA. The decrease of thymidine incorporation is not due to a cytotoxic effect of the α2H globulin.

TABLE 4

EFFECT OF INCREASING DOSES OF α2H GLOBULIN UPON PHA-INDUCED INCORPORATION OF [^{3}H]THYMIDINE, IN NORMAL HUMAN LEUCOCYTES

Case number		Spontaneous transformation (ct/minute)	PHA-induced transformation (ct/minute)	PHA-induced transformation in the presence of 2–1000 μg/ml of α2H globulin							
				2	5	25	50	100	200	500	1000
18	Ct/minute	2061	123,826	62,800	63,641	23,776	12,708	9335	4575	3005	3001
	I_s			0·51	0·51	0·19	0·10	0·08	0·03	0·02	0·02
19	Ct/minute	1296	60,413	29,494	24,513	15,445	13,636	12,056	2431	2903	3012
	I_s			0·45	0·40	0·25	0·22	0·19	0·04	0·04	0·05
41	Ct/minute	485	112,917	82,073	71,931	34,311	20,015	12,036	8218	5882	3010
	I_s			0·72	0·69	0·30	0·19	0·10	0·07	0·05	0·02
42	Ct/minute	739	61,203	45,907	40,404	18,267	11,179	10,122	3550	3126	1786
	I_s			0·75	0·66	0·29	0·18	0·16	0·05	0·05	0·02
43	Ct/minute	1027	59,453	42,316	37,102	18,430	14,268	11,729	6539	4675	2792
	I_s			0·71	0·62	0·31	0·23	0·19	0·10	0·08	0·05

TABLE 5

PHA + α2H GLOBULIN-INDUCED TRANSFORMATION OF NORMAL LYMPHOCYTES JUDGED BY [^{3}H]THYMIDINE INCORPORATION AFTER 5 DAYS OF CULTURE

α2H added (μg/ml)	Without pre-incubation with α2H		After 24 hours of preincubation with α2H globulin		
	Ct/minute	I_s	Percentage decrease in I_s	I_s	Ct/minute
0	61,203				61,203
2	45,907	0·75	10	0·67	41,616
5	40,404	0·66	18	0·54	33,313
25	18,267	0·29	28	0·22	13,906
50	11,178	0·18	27	0·14	8568
100	10,122	0·16	37	0·10	6378
200	5550	0·09	44	0·05	3102
500	3126	0·05	60	0·02	1308
1000	1786	0·02	50	0·01	1113

When the lymphocytes were cultivated in the presence of the purified α2H globulin alone, the thymidine incorporation was often higher than the spontaneous incorporation, demonstrating in these experiments a stimulating effect (Table 6). To emphasize this point, we have determined an index of α2H activity:

$$I\ \alpha2H = [(\text{ct/minute in presence of } \alpha2H \text{ globulin})/(\text{spontaneous ct/minute})].$$

If this index was between 0·8 and 1·5, we concluded that the globulin had no effect on the culture. We considered α2H to have a stimulating activity when the index was greater than 1·5 and a toxic effect if the index was less than 0·8. (Table 7).

As shown in Table 8 100 μg/ml of α2H globulin had a lymphocytotoxic action in two out of forty-six cases (4·3 per cent) and a stimulating effect in twenty-four out of forty-six cases (52·7 per cent).

TABLE 6

In vitro INCORPORATION OF [^{3}H]THYMIDINE IN NORMAL LYMPHOCYTES IN THE PRESENCE OF PHA AND α2H GLOBULIN. DETERMINATION OF THE INDEX OF α2H ACTIVITY

Case number	Spontaneous transformation (ct/minute)	Index of PHA transformation $\left(\frac{\text{ct/minute PHA}}{\text{ct/minute (spontaneous)}}\right)$	α2H-Induced transformation (ct/minute)	Index of α2H activity $\left(\frac{\text{ct/minute α2H (100 μg/ml)}}{\text{ct/minute (spontaneous)}}\right)$
1	1423	58·25	3746	1·94
2	511	69·32	973	1·90
3	493	83·83	507	1·02
4	725	84·85	5375	7·41
5	895	125·50	749	0·83
6	913	132·61	4731	5·18
7	580	79·46	602	1·03
8	327	76·24	979	2·99
9	2340	53·83	1509	0·64
10	613	14·91	895	1·46
11	1461	5·8	3372	2·30
12	1189	132·05	10,162	8·50
13	473	129·23	1237	2·61
14	1192	81·67	1471	1·23
15	531	113·09	427	0·80
16	1826	69·55	30,316	16·00
17	903	53·4	1979	2·19
18	2061	60·08	9172	4·40
19	1296	46·61	25,089	19·20
20	1784	68·21	1819	1·00
21	1007	53·22	592	0·58
22	1826	65·78	2023	1·10
23	458	103·94	864	1·88
24	948	50·87	931	0·98
25	1501	75·17	3709	2·47
26	1219	58·6	4436	3·63
27	478	10·83	501	1·04
28	503	11·03	419	0·83
29	619	92·86	831	1·34
30	356	104·73	1052	2·95
31	252	132·52	1103	4·37
32	2137	42·58	3010	1·40
33	534	95·51	7931	14·90
34	1155	57·13	1616	1·39
35	1754	75·42	2597	2·95
36	376	101·84	1732	4·60
37	607	77·99	1001	1·64
38	1071	55·05	1533	1·43
39	963	55·03	994	1·03
40	1268	64·98	1014	0·79
41	485	232·8	20,543	4·24
42	739	82·2	33,201	3·98
43	1027	57·8	83,201	32·30
44	9380	2·33	10,007	1·06
45	386	2·9	627	1·62
46	13,971	2·02	11·181	0·80

α2H globulin is a ferroprotein and the different fractions used for these experiments were more or less rich in iron. The α2H globulin rich in iron (15 per cent of the molecule) and the α2H globulin poor in iron (2–5 per cent) or even the iron-free fraction had the same inhibitory effect on the PHA induced incorporation within the limits of technical variation.

Nevertheless, to eliminate the possible stimulating role of ionic iron on blastic transformation, we tested the PHA stimulation of tritiated thymidine incorporation in the

TABLE 7

REFERENCE VALUE OF THE α2H ACTIVITY: Iα2H = [(CT/MINUTE IN THE PRESENCE OF α2H GLOBULIN)/(CT/MINUTE IN THE MEDIUM (SPONTANEOUS INCORPORATION))]

$I_{\alpha 2} < 0{\cdot}8$	Toxic action
$0{\cdot}8 < I_{\alpha 2} < 1{\cdot}5$	No effect
$I_{\alpha 2} > 1{\cdot}5$	Stimulating action

TABLE 8

Index value	Number of cases	Percentage
$I_{\alpha 2} < 0{\cdot}8$	2	4·3
$0{\cdot}8 < I_{\alpha 2} < 1{\cdot}5$	20	43
$I_{\alpha 2} > 1{\cdot}5$	24	52

presence of $FeCl^3$ (5×10^{-4}M). This concentration of iron, which is higher than the concentration of the richest fraction of α2H globulin, did not modify the incorporation of [^{3}H]thymidine into lymphocyte DNA.

II. STUDY OF LYMPHOBLASTIC TRANSFORMATION AS JUDGED BY MICROSCOPIC EXAMINATION

On day 6, 0·5 ml of lymphocytic suspension was harvested. The cells were spread on slides and stained with May–Grünwald–Giemsa stain.

The microscopic aspect of the lymphoblastic transformation was observed as follows.

When the cells were cultured with PHA 65–80 per cent of large mononucleated cells (20–30 μm in diameter) were seen. The nucleus of these cells is large, with or without an obvious nucleolus. Generally, the chromatin is finely structured, but may be very loose and pale, especially in the largest cells. The cytoplasmic rim is extensive and basophilic and often it contains unstained vacuoles. These cells correspond to those considered as lymphoblastic cells. Some mitoses were also seen on these slides.

In the reference cultures without PHA similar cells were observed, but less than 8 per cent.

The morphological aspect of the cells cultured in the presence of α2H globulin was very different. There was no typical blastic transformation. Most of the cells seemed to be small lymphocytes, often smaller than normal lymphocytes with a diameter of 5–10 μm. The chromatin of the nucleus was very dense and stained black; no nucleolus was seen. The nucleus occupied almost the whole cell; the cytoplasm was reduced to a very thin rim around the nucleus. Some of these cells were joined together by narrow cytoplasmic bridges forming small chains. We did not observe mitosis in these microscopic preparations.

DISCUSSION

α2H globulin is a macroprotein which is normally localized intracellularly in the liver (Rimbaut, 1974). In the blood stream of healthy subjects it is found only in trace quantities but its synthesis is increased in some pathological disorders, especially in malignant diseases.

In these cases, this protein is found in the sera in quantities which could have an inhibitory effect on the lymphocytic response as judged by the response to the PHA stimulation *in vitro*. In fact, the inhibitory action is very effective at doses as low as 5 μg/ml, levels frequently observed in sera of patients with tumours.

α2H globulin could be one of the components of the inhibitory serum fractions rich in proteins of α2 electrophoretic mobility reported by Kamrin (1959), Mowbray (1963) or could be the IRA (immunoregulatory globulin) described by Cooperband *et al.* (1968). These authors do not mention the presence of iron in their fractions but they mention the carbohydrate component. Riggio *et al.* (1971) have reported a similar activity of a solubilized glycoprotein fraction extracted from human placenta. We know that the placenta is one of the organs rich in α2H globulin.

That α2H globulin is one of these effective glycoproteins still requires verification by exchange of material. Some properties of these substances are not identical; Riggio's fraction, extracted from placenta is a perchloric acid-soluble material and although α2H globulin is also a polydisperse protein, most of it is insoluble in 0·6 N perchloric acid. Our hepatic globulin has no ribonuclease activity, as did Mowbray's fraction.

The response of lymphocytes to PHA is thought to be characteristic for thymus-dependent cells (Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969; Janossy and Greaves, 1971). If α2H globulin is a potent inhibitor of the immunological mechanism of T cells, it would have to block a given antigen response known to be associated with delayed hypersensitivity and spontaneous rosette formation.

Preliminary results showed that α2H globulin was able to inhibit *in vitro* PPD tuberculin- and candidin-induced stimulation of sensitized donors' lymphocytes.

The presence of α2H globulin in the blood could explain the anergy of some patients with carcinoma or other malignant diseases that has been reported by many authors (Trubowitz, Masek and Del Rosario, 1966; Gatti, Garioch and Good, 1970; Gatti, 1971; Nairn, Nind, Guli, Davies, Rolland, McGiven and Hughes, 1971a; Nairn, Nind, Guli, Muller, Roland, and Minty, 1971b; Nairn, Nind, Guli, Davies, Little, Davis and Whitehead, 1972; Whittaker, Rees and Clark, 1971; Nind, Nairn, Roland, Guli and Hughes, 1973). The increasing level of α2H globulin observed in the serum, a few weeks before metastasis, would then be responsible for the spread of the disease by blocking cellular immune defences. These changes would be one of the consequences of hepatic metabolic disorders as the modifications frequently and precociously observed in the serum of patients with tumours. (Rimbaut, Tilz and Buffe, 1974).

We are comparing the cutaneous reactivity of patients with the level of α2H globulin in their sera in order to verify a possible parallelism between delayed cutaneous hypersensitivity and serum α2H level.

The mechanism of activity of α2H globulin on lymphocytes may be explained by the nature of the protein. α2H globulin is a glycoprotein which adheres to the cell membrane and coats it; in this way it can block the sites responsible for antigen recognition.

The results obtained with PHA-stimulated lymphocytes in the presence of α2H globulin or after preincubation with this hepatic protein are in agreement with this hypothesis; competition would be between PHA and α2H globulin.

On the other hand, because α2H is a macroglobulin, it may trigger non-specifically DNA synthesis when it is bound to the membrane; in fact increased tritiated thymidine incorporation was found with α2H globulin alone in the culture medium, without mitogen or antigen.

The effect of α2H globulin does not seem to be specific for T cells as shown by other results (Tilz, Rimbaut and Buffe, in preparation) on spontaneous rosette formation.

Morphological examination of the lymphocytes incubated with α2H globulin shows that, if there is an increase in DNA synthesis as judged by incorporation of [^{3}H]thymidine and density of the nuclear chromatin, it is not a proper lymphoblastic transformation. Indeed, the first step of the lymphocyte transformation, after the agglutination of the cells, is enlargement of the cytoplasm and increase of synthesis of ribonucleic acids and cellular proteins necessary to the cell structure and especially to form the spindle on which the chromosomes migrate during mitosis.

In the presence of α2H globulin there is no aggregation of cells and no cytoplasmic enlargement. The absence of mitosis on day 6 and the presence of abnormal small chains of cells could be due to a defect in protein metabolism. α2H Globulin may block or modify cell respiration. The mechanism of activity may be not only an immunosuppressive effect, but a more general phenomenon, and we are investigating this on different types of cells in tissue culture.

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