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Demonstration of Inhibitory Factors Affecting Cell-Mediated Immunity in Patients with Hodgkin's Disease*

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Abstract—*In the present investigations attempts have been made to study factor(s) involved in impairment of cellular immunity in Hodgkin's disease (HD). Peripheral blood lymphocytes from* 42 *untreated HD patients in differnent stages of the disease showed depressed PHA responsiveness as compared to* 23 *mormal healthy individuals. Peripheral blood lymphocytes of* 7/13 *HD patients showed partial restoration of ability to respond to PHA after overnight incubation in tissue culture media, supplemented either with human AB serum or fetal calf serum (FCS). The restoration appeared to be more common in cultures incubated in media supplemented with FCS than with AB serum. Supernatants of lymphocyte cultures from HD patients and their sera showed significant inhibition of PHA induced transformation of lymphocytes obtained from healthy donors. In contrast, normal lymphocyte culture supernatants and sera were less inhibitory to PHA induced transformation of normal lymphocytes. Cultures of HD lymphocytes, which failed to restore PHA responsiveness after overnight incubation, also showed inhibitory factors in supernatants.*

INTRODUCTION

HODGKIN'S disease is known to be associated with impaired cell mediated immunity (CMI), which is characterised by diminished delayed hypersensitivity [1, 2], impaired ability to reject homografts [3], decreased percentage of SRBC rosette forming cells [4, 5], depressed response to mixed lymphocyte reaction [6] and impaired ability to respond to mitogens and antigens [7–10]. Whether this depressed state of CMI in patients with HD is due to an intrinsic defect of T-lymphocytes [10, 11] or it is an acquired deficiency due to diseased state [7] is not yet clear. Humoral factors from sera of HD patients have been shown to interfere with the receptors for SRBC on T-lymphocytes [12, 13] and perhaps with their functional ability also [14, 15].

In the present investigation, untreated patients with HD have been assessed for their cellular immunity. The ability of peripheral blood lymphocytes of these patients to respond to PHA and to form E and EAC rosettes has been studied. The peripheral blood lymphocytes of HD patients have been further subjected to incubation in tissue culture medium for 18–24 hr before reassessing their ability to respond to mitogen. The lymphocyte culture supernatants and sera from HD patients have been tested for their inhibitory effect on PHA induced transformation of lymphocytes obtained from healthy donors.

MATERIALS AND METHODS

Untreated patients with HD selected for studies were in all stages of disease varying from stage I to stage IV. As controls, healthy individuals from medical and paramedical staff were selected.

Five ml aliquots of peripheral blood from HD patients and normal healthy donors were collected in 0.5 ml heparin (100 i.u./ml) and lymphocytes were separated on Ficoll–Hypaque gradient as described earlier [16].

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Separated lymphocytes were washed thrice with 0.85% saline and were suspended in RPMI 1640 supplemented with 10% FCS (Difco Labs., Michigan) at the concentration of 1×10^6 cells/ml for PHA responsiveness and 2×10^6 cells/ml for rosette tests.

Rosette test

E and EAC rosettes were performed according to the method described by Jondal *et al.* [17]. Peripheral blood lymphocytes from 30 HD patients and 17 healthy donors were tested for their ability to form EAC rosettes, while SRBC rosette forming cells were evaluated in 31 HD patients and 21 healthy donors. E rosettes were counted after overnight incubation at 4°C.

Lymphocyte blastogenesis

This test was carried out in microtubes, in which 0.2 ml of 1×10^6 cells/ml in RPMI 1640 + 10% FCS were incubated with or without PHA. (Wellcome, 10 μl of 1:10 dilution) at 37°C in humidified 5% CO_2 atmosphere. To each of the microtube cultures 0.05 μCi of ^{3}H-TdR (sp. act. 6–9 mCi/mM, BARC, Bombay) was added 16 hr before harvesting. Cultures were harvested at 72 hr on Whatmann No. 3 filter paper discs and processed for scintillation counting as described before [16]. Results are expressed in blastogenic index (B.I.) where,

$$\text{B.I.} = \frac{\text{Absolute counts/min in PHA-treated cultures}}{\text{Absolute counts/min in untreated cultures}}$$

Lymphocytes from 13 patients (2×10^6 cells/ml) were incubated in RPMI 1640 supplemented with either 10% human AB serum or FCS, at 37°C in humidified 5% CO_2 atmosphere for 18–24 hr. After incubation cells were centrifuged, resuspended in fresh medium and tested for their PHA responsiveness. Supernatants from these cultures were separately collected and stored at −20°C until tested for their effect on PHA responsiveness of normal lymphocytes. Sera from HD patients were collected before therapy, inactivated at 56°C for 30 min and stored at −20°C. They were also tested for their effect on PHA responsiveness of normal lymphocytes. Control tests were performed with sera from healthy donors and supernatants of normal lymphocyte cultures.

As described before, microcultures were set-up to test the effect of normal and HD lymphocyte culture supernatants and sera on PHA induced transformation of normal lymphocytes. To 0.2 ml of lymphocyte suspension (1×10^6 cells/ml) dispensed in microtubes, 50 μl of serum/culture supernatant from normal/HD individuals was added in duplicate, along with 10 μl of PHA. These cultures were termed as "treated cultures". Another set of cultures with PHA but without addition of normal/HD serum or supernatant, served as controls, which were termed as "untreated cultures". Blastogenic indices of all the cultures were calculated as described before and percentage inhibition in PHA responsiveness was recorded as follows:

$$\text{Percentage inhibition} = \frac{\text{B.I. of untreated cultures} - \text{B.I. of treated cultures}}{\text{B.I. of untreated cultures}}$$

Inhibition $\geqslant 50\%$ was considered as positive [18].

RESULTS

Rosette assay

Scattergram in Fig. 1. shows the percentage of E-rosette forming cells (E-RFC) and EAC-rosette forming cells (EAC-RFC) in peripheral blood of HD patients and healthy

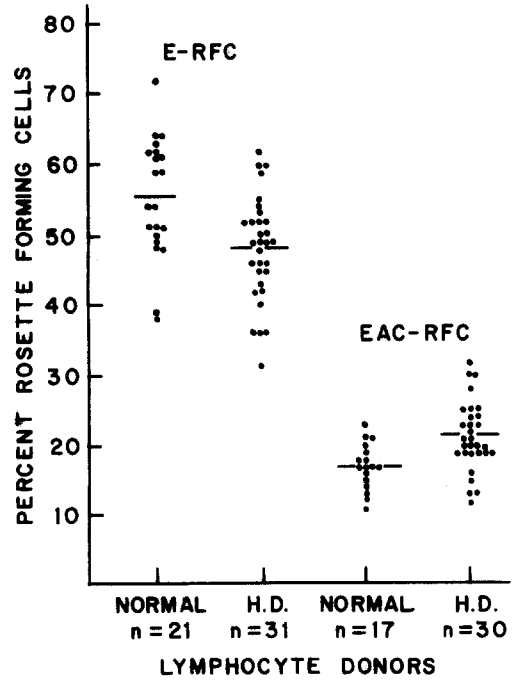

Fig. 1. Percentage of E and EAC rosette-forming cells in peripheral blood lymphocytes of untreated HD patients and healthy donors.

donors. Percentage of E-RFC in HD patients was found to be decreased as compared to the normal controls but this decrease was not statistically significant when assessed by Student's *t*-test. Percentage of EAC-RFC was found to be marginally increased in HD patients than the normals. This difference also was not statistically significant.

Lymphocyte blastogenesis

Scattergram in Fig. 2. reveals PHA responsiveness of peripheral blood lymphocytes of HD patients as compared to that of healthy donors. PHA induced transformation was found to be depressed in more than 80% of the patients.

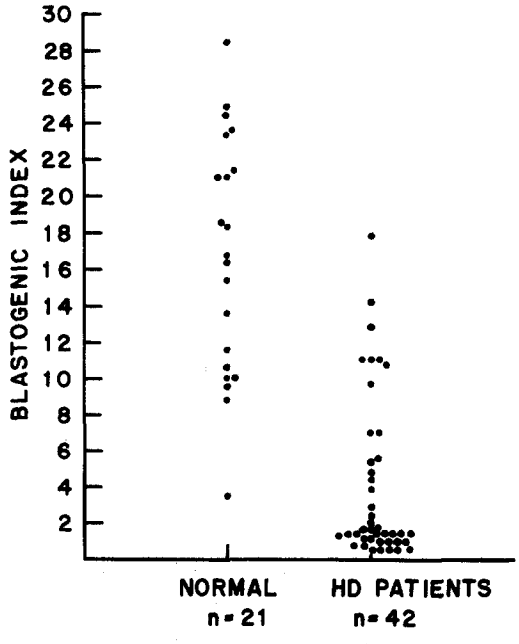

Fig. 2. Blastogenic response of lymphocytes to PHA from untreated HD patients and healthy donors.

Effect of incubation of HD lymphocytes in tissue culture medium on their PHA responsiveness

Out of 42 patients tested for their PHA responsiveness, 13 were studied for PHA responsiveness of lymphocytes before and after incubation in tissue culture medium. These results are shown in Table 1. Out of 13 patients, 6 showed more than 50% increase and one showed nearly 50% increase in PHA responsiveness after incubation in RPMI 1640 +10% FCS while only one patient out of nine showed increased PHA responsiveness after incubation in RPMI 1640+10% AB serum. However, this recovery was not comparable to the PHA responses of lymphocytes from healthy donors, the mean B.I. of healthy donors' lymphocytes being 17.36±11.04 and that of recovered HD lymphocytes being 9.29 ±8.01.

Effect of culture supernatants and sera from HD patients and normal donors on PHA responsiveness of normal lymphocytes

Culture supernatants of lymphocytes from 14 HD patients, incubated in RPMI 1640 +10% AB serum and from 17 HD patients, incubated in RPMI 1640+10% FCS were tested for their inhibitory effect on PHA responsiveness of normal lymphocytes from healthy donors. The

Table 1. PHA responsiveness of lymphocytes from patients with HD before and after incubation in tissue culture medium

No.	Patient	Blastogenic index Without preincubation	Preincubation with RPMI 1640+10% human AB serum	Preincubation with RPMI 1640+10%FCS
1.	AD 1208	5.49	17.7*	11.54*
2.	AJ 16109	7.05	—	15.45*
3.	AK 615	1.25	—	6.01*
4.	AK 2050	7.04	—	7.78
5.	AK 2506	2.94	1.62	17.30*
6.	AK 2704	9.70	1.37	5.75
7.	AK 3235	0.74	1.01	3.65*
8.	AK 3272	4.41	—	8.62*
9.	AK 3482	1.35	1.40	0.87
10.	AK 4519	1.46	1.11	0.93
11.	AK 4892	0.92	0.95	0.95
12.	AK 5139	1.20	0.83	2.45*
13.	AK 6080	1.05	1.22	1.76

*Restoration of PHA responsiveness.

number of tests carried out were 58 and 68, respectively. As controls culture supernatants from eight normal lymphocyte samples incubated in AB serum medium and from four normal lymphocyte samples incubated in FCS medium were tested for their effect on PHA responsiveness of allogeneic normal lymphocytes in 12 and 13 tests respectively. The data are represented as a scattergram in Fig. 3. Also included in Fig. 3 is the inhibitory/facilitating effect of sera obtained from 14 HD patients and eight normal donors; the number of tests performed being 65 and 34, respectively. The results are summarised and statistically evaluated in Table 2. Inhibition in PHA responsiveness to $\geqslant$ 50% level has been considered as significant [18]. It can be seen that lymphocyte culture supernatants of HD patients, supplemented with human AB serum as well as FCS, and HD patients' sera show significant inhibition of PHA responses of normal lymphocytes.

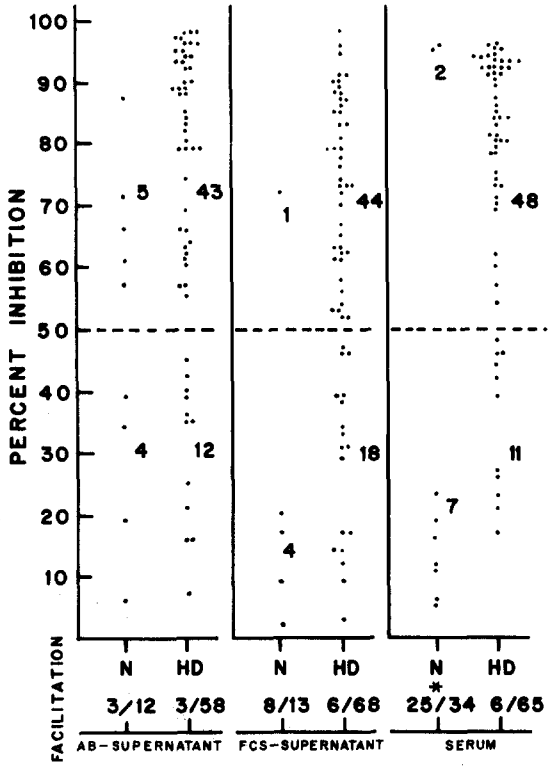

Fig. 3. Effect of normal/HD lymphocyte culture supernatants and sera on PHA responsiveness of normal lymphocytes. *14/25 *tests showed more than* 100% *facilitation.*

It was interesting to note that 3/12 and 8/13 tests carried out with AB and FCS supernatants of normal lymphocytes cultures, respectively, and 25/34 tests with normal sera showed facilitory effect on normal lymphocyte transformation. Compared to these, very few tests with HD supernatants (3/58 with AB and 6/68 with FCS) and sera (6/65) showed facilitory effect on normal lymphocyte transformation. If 100% increase in blastogenic response is considered significant facilitation as suggested by Mavligit *et al.* [18] then normal serum samples alone showed significant facilitation of normal lymphocyte transformation in as many as 14 tests.

As mentioned earlier in Table 1, lymphocytes from only 1/9 patients showed restoration of ability to respond to PHA after incubation in medium containing human AB serum. It was therefore important to study whether the supernatants of "nonrestorable" lymphocyte cultures were also inhibitory to normal lymphocyte transformation. Table 3 gives the data on tests carried out with nine lymphocyte culture supernatants from HD patients mentioned in Table 1. Each culture supernatant was tested on four normal lymphocyte samples. It can be clearly seen from the table that the majority of the supernatants tested show $\geqslant$50% inhibition of PHA response of normal lymphocytes. Inhibitory factors, apparently were detected even in those lymphocyte culture supernatants, where the preincubated lymphocytes were unable to show restoration of PHA responsiveness.

DISCUSSION

The results obtained in our studies are in agreement with the well established fact that patients with HD often show depressed response to PHA [7, 8]. However, we did not get significant reduction in ERFC. There are controversial reports regarding the percentage

Table 2. *Comparison of inhibitory effect of lymphocyte supernatants and sera from healthy individuals and HD patients*

Tests carried out with	Percentage of tests showing $\geqslant$50% inhibition Normal	HD	*P* value by x^2 analysis
AB Supernatants	41 (5/12)	74 (43/58)	<0.05
FCS Supernatants	8 (1/13)	65 (44/68)	<0.001
Sera	5 (2/34)	74 (48/65)	<0.001

Table 3. Effect of supernatants obtained from HD patients' lymphocytes on PHA responsiveness of normal lymphocytes

Supernatants from HD patients	Percentage inhibition in B.I. of normal lymphocytes when tested with AB supernatants				FCS supernatants			
	I	II	III	IV	I	II	III	IV
AD 1208*	97	79	12	61	57	88	62	38
AK 2050	56	73	38	65	89	74	52	46
AK 2506	–	–	–	–	21	−20	12	–
AK 2704	95	93	−10	89	85	69	64	85
AK 3235†	89	89	20	78	90	87	91	90
AK 3482	98	96	90	85	89	79	64	46
AK 4892	88	64	13	60	53	56	65	–
AK 5139†	98	83	64	85	32	54	61	–
AK 6080	95	81	94	–	82	60	33	–

*Patient showing restoration of PHA response of lymphocytes after incubation in both FCS and AB serum supplemented medium (Table 1).

†Patients showing restoration of PHA response of lymphocytes after incubation in FCS supplemented media alone (Table 1).

of T-cells in circulation in HD patients. Kaur *et al.* [19] and Swain and Trounce [20] have observed normal levels of ERFC in peripheral blood of untreated HD patients. Fuks *et al.* [21] have reported impaired ability of lymphocytes from HD patients to form E-rosettes, which was attributed to altered cell surface receptors. This defect however, was not found to be permanent. Marginal increase in percent EAC-RFC found by us could be because of elevated number of monocytes [22] in HD peripheral blood which also form EAC rosettes.

Impaired cellular immunity in HD patients has been much debated so far. Twomey *et al.* [6] suggested that immune deficiency with advanced HD could be due to depletion of T-lymphocytes and it might be compounded with a relative excess of suppressor lymphocytes. It could also be argued that immunodeficiency could be because of direct involvement of immunocompetent cells in neoplastic process. As suggested by Goodwin *et al.* [23], the suppressor cell products such as prostaglandin might be directly inhibitory to circulating immunocompetent cells. The impaired CMI could be due to cell mediated suppression by suppressor T-cells and monocytes as suggested by Hillinger *et al.* [24]. Plasma/serum from HD patients affecting PHA responsivenesss [15] and E-rosette forming ability of T-lymphocytes [12, 13] has been reported earlier. Spontaneous release of an inhibitor from HD lymphocytes to normal lymphocyte transformation has been reported by Golding *et al.* [25]. Han [14] however, was unable to demonstrate the presence of inhibitory factor in serum of HD patients; and in untreated cultures felt that it may not be an important cause of immunodeficiency in Hodgkin's disease.

In the present investigation we have confirmed that PHA responsiveness of HD patients' peripheral blood lymphocytes was depressed, although the T-cell number *per se* did not seem to be reduced significantly. We have also observed that if non-reactive lymphocytes were incubated in tissue culture media for 18–24 hr, some of them showed partial restoration of PHA responsiveness. Supernatants from these cultures and sera of HD patients showed inhibition of PHA responses of lymphocytes from healthy donors.

These observations suggest two possibilities. Firstly, soluble humoral factors, responsible for immunosuppression may be nonspecifically adhering to lymphocytes, which are released in the median after incubation. These soluble inhibitors may be the products of lymphocytes themselves or suppressive factors like prostaglandin produced by monocytes as suggested by Goodwin *et al.* [23]. Serum inhibitory factors, capable of suppressing PHA responsiveness of normal lymphocytes have been demonstrated in the sera of HD patients earlier [15]. Our experiments have added further information along this line. We have been able to demonstrate the presence of inhibitory factors affecting PHA transformation of normal lymphocytes in sera as well as in lymphocyte culture supernatants. Fuks *et al.* [21] have demonstrated restoration of surface markers of HD lymphocytes after overnight incubation as shown by their increased ability to form E-rosettes. They have also put forward an evidence suggesting restoration of

functional ability of HD lymphocytes after incubation, by evaluating their PHA responsiveness. Our results confirm their findings and suggest further that HD patients' lymphocytes, after incubation, not only acquire the ability to respond to PHA but also release the suppressive factors in the medium.

Secondly, it is also possible that during the process of overnight incubation, monocytes which may be acting as suppressors for *in vitro* PHA responsiveness [22] may have got attached to the glass surface of microtubes, thus leaving a partially monocyte depleted lymphocyte population, which could respond to PHA. Depletion of monocytes might render the lymphocyte population free of influence of monocyte products such as prostaglandin which have been implicated in immunosuppression [23, 24].

During the course of these investigations it was noted that partial restoration of PHA responsiveness was more common in HD lymphocyte cultures supplemented with FCS than with human AB serum. Fuks *et al.* [21] have suggested that reversal of cell surface abnormality in HD is not merely mediated by shedding of the extrinsic factors bound to the cell surface but rather by complex interaction of components in FCS with extrinsic factors and perhaps with the receptors on T-cells. However, although AB serum supplemented lymphocytes were, after incubation, unable to respond to PHA; the inhibitory factors were apparently released into the medium, as these supernatants also inhibited normal PHA response.

It is evident from the scattergram in Fig. 3 that some of the supernatants from normal lymphocyte cultures were also inhibitory to PHA responsiveness of normal allogeneic lymphocytes. We have therefore not compared the effect of one HD lymphocyte culture supernatant with one normal lymphocyte culture supernatant on a given lymphocyte sample. The overall pattern of the tests independently carried out with HD lymphocyte culture supernatants/sera and normal lymphocyte culture supernatants/sera showed statistically significant inhibition by HD factors.

Facilitation of PHA response, shown by few normal human sera could be attributed to better growth conditions for these cultures because of additional serum supplementation which lacked inhibitory factors.

The nature of the factors involved in bringing about inhibition of PHA responsiveness of normal lymphocytes needs to be elucidated further with special reference to lymphocyte/monocyte products.

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