In-vitro T cell mediated function in patients with active rheumatoid arthritis

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SUMMARY In-vitro protein synthesis of peripheral blood lymphocytes from patients with rheumatoid arthritis was measured after stimulation with phytohaemagglutinin (PHA) in a short-term, serum-free culture system. Diminished responses were found in 16 out of 17 consecutive patients with active disease. Normal PHA responsiveness was recovered by assaying Ficoll-Hypaque isolated E rosette forming cells in serum-free medium, indicating basically normal T cell function in RA. Preincubation of normal peripheral blood lymphocytes (or isolated E rosette forming cells) with sera obtained from patients with active RA for 30 minutes at 4°C or 37°C blocked PHA responsiveness in 34 out of 43 tests. This suggests that serum blocking factors may be responsible for reduced T cell reactivity in RA.

Rheumatoid arthritis (RA) is a disease of unknown aetiology characterised by persistent immunological abnormalities including chronic synovitis, granulomata, and autoantibodies directed against the constant region of IgG. Studies of the thymus-dependent (T) cell mediated immune functions in RA have revealed inconsistent responses from one laboratory to another as well as great variability between different individual patients.¹⁻⁶ Defects in the function of lymphocyte subpopulations may contribute to the pathogenesis of RA in any one of a few pathways involving the helper or suppressor role of T lymphocytes, leading to an inadequate response or to uncontrolled hyperresponsiveness, respectively. It is possible that different in-vitro T cell responses characterise different subsets of RA patients. Diminished T cell mediated immune responsiveness cannot be attributed to low T cell number,⁵ which suggests that there is either a derangement in T lymphocyte subpopulations or factors that block T lymphocyte responsiveness.

The purpose of the present work was to study the mechanism of diminished in-vitro immune reactivity of lymphocytes obtained from patients with RA. We assayed protein synthesis of phytohaemagglutinin (PHA) stimulated lymphocytes and

found that the PHA responsiveness of unseparated lymphocytes was usually diminished. However, a normal response was recovered by assaying purified T cells. PHA responsiveness could be blocked after a short preincubation of normal lymphocytes with sera obtained from patients with active disease, suggesting that serum factors play an important role in diminishing T cell reactivity in RA.

Materials and methods

PATIENTS

Seventeen consecutive patients with active classical RA were studied, including 6 male and 11 female patients. Sera were stored at 70°C until used.

LYMPHOCYTE STIMULATION BY PHA

Blood was collected into preservative-free heparin-coated glass tubes. Lymphocytes were isolated in a Ficoll-Hypaque gradient (40 minutes, room temperature at 400 g⁷ and were washed 3 times in phosphate buffered saline (PBS) and diluted to a final concentration of 2.5×10^6 viable cells/ml in leucine-free minimum essential medium (MEM) supplemented with 1% nonessential amino acids, glutamin (2mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Viability of cells was checked by a trypan blue exclusion assay. No serum was added at any time. Cell suspensions containing 0.2 ml V-bottom

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microtitre wells (Cooke Laboratories Products Division, Dynatech Laboratories Inc., Alexandria, Va) followed by the addition of 0.02 ml purified PHA (Burroughs Wellcome Company, London at final concentrations of 2.5 and 5.0 µg/ml. Equal amounts of PBS were added to the controls. After 20 hours of incubation at 37°C in a humidified atmosphere of 5% CO₂ in air, 2 µCi in 0.01 ml ³H-leucine (New England Nuclear, NET 135 H, Boston, Mass) was added for 90 minutes. Cells were subsequently collected on paper filters by a multiple sample harvester (Mass II, Microbiological Associates Inc., Bethesda, Md), and ³H uptake was read in a liquid scintillation counter. All cultures were performed in triplicate. The data are expressed as the ratio of ³H counts incorporated in the presence of PHA as compared with the simultaneous incorporation of ³H in controls.

BLOCKING OF PHA RESPONSIVENESS BY RA SERA

Aliquots of cells were delivered into microtitre plates as described above at a concentration of 5×10^5 cells/0.1 ml with either 0.05 ml normal AB serum or 0.05 ml serum samples obtained from patients with active RA for 30 minutes at 40°C or 37°C. All sera were inactivated at 56°C for 30 minutes. After incubation the cells were washed twice in PBS and resuspended in 0.2 ml MEM medium as described above.

Results were compared to PHA responses obtained by cells incubated in serum-free medium.

IDENTIFICATION OF LYMPHOCYTE

SUBPOPULATIONS AND ISOLATION OF T CELLS
T lymphocytes were identified by a complement-dependent microcytotoxicity assay.⁸ This method utilises an anti-T cell antiserum developed by immunising an adult goat with viable human thymus cells and subsequent absorption of the crude antiserum with malignant B cells. The percentage of cells killed by the antiserum was determined by trypan blue exclusion. T lymphocytes were also identified by their ability to form spontaneous rosettes with sheep erythrocytes (E rosettes).⁹ At least 200 cells were counted, and each test was done in triplicate. Only lymphocytes with at least 3 erythrocytes attached were considered E rosette forming cells.

B lymphocytes were identified by staining the Ig-bearing cells with a fluorescein-conjugated polyvalent rabbit antihuman Ig antiserum.⁸

Monocyte contamination was determined by staining Ficoll-Hypaque purified cells with alpha-naphthol acetate (Sigma Chemical Co., St. Louis, Mo).¹⁰ The percentage monocyte contamination

and the cytotoxic index obtained with the anti-T cell serum was used to calculate the percentage of T cells in the peripheral blood as follows:

$$\% \text{ T cells} = \text{cytotoxic index (\%)} \times \frac{100}{100 - \% \text{ monocyte contamination}}$$

The percentage of B cells was independent of the monocyte contamination, since only small cells were examined for surface Ig staining, thereby excluding monocytes.⁸

T cells were isolated by layering E rosette forming cells over a Ficoll-Hypaque gradient and collecting the sediment. Sheep erythrocytes were lysed by ammonium chloride.

Results

RESPONSE OF PERIPHERAL BLOOD LYMPHOCYTES FROM PATIENTS WITH RA TO PHA

The PHA responsiveness of unseparated lymphocytes at 2 concentrations was compared to that of Ficoll-Hypaque isolated E rosette forming T cells ($\geq 90\%$ purity) and the B cell enriched fraction (70% purity). The response (cpm ³H-leucine uptake of PHA-stimulated/cpm ³H-leucine of unstimulated cells) was significantly lower than the lowest range of normal responses in 16 out of 17 cases with active RA (Table 1). Isolated E rosette forming cells from all 8 RA patients examined, including those with lowest PHA responsiveness, showed normal responses as compared to normal T cells, with a significant increase over that observed in unseparated lymphocytes (Table 1). The response of the B enriched fraction to PHA was low and similar to that observed in normal individuals (Table 1).

PROPORTIONS OF LYMPHOCYTE SUBPOPULATIONS IN RA

The percentage of T cells in RA was in the normal range as measured by the E rosette assay ($60 \pm 7\%$) as well as by a cytotoxicity assay using a highly specific anti-T cell serum ($80 \pm 11\%$) (Table 2). No variation was observed in the proportions of Ig-bearing (B cells) in the blood between normal and RA patients (Table 2).

INHIBITION OF NORMAL LYMPHOCYTE REACTIVITY TO PHA BY SERA OBTAINED FROM PATIENTS WITH ACTIVE RA

Sera from patients with active RA inhibited the normal lymphocyte response to PHA. Ficoll-Hypaque purified lymphocytes from 10 normal individuals were preincubated for 30 minutes at 37°C and 4°C in heat-inactivated sera (50% v/v)

Table 1 *Phytohaemagglutinin (PHA) stimulation of protein synthesis in peripheral blood lymphocytes and isolated E rosette forming cells of patients with active rheumatoid arthritis (RA) and normal controls. The data are expressed as the stimulation ratio of ³H-leucine uptake by PHA-stimulated cells to unstimulated controls*

	PHA concentration			
	2.5 µg/ml		5.0 µg/ml	
	(Mean stimulation index ± SE)			
	RA	Normals	RA	Normals
Unseparated mononuclear cells*	1.71 ± 0.23	3.29 ± 0.29	2.20 ± 0.21	4.88 ± 0.26
E-rosetting cells†	4.25 ± 0.28	4.38 ± 0.42	5.45 ± 0.20	5.76 ± 0.33
Non E rosetting cells*	1.11 ± 0.10	1.12 ± 0.11	1.23 ± 0.10	1.39 ± 0.14

*Data based on tests done in 17 RA patients and 20 normal controls.

†Data based on tests done in 8 patients with RA and 8 normal controls.

Table 2 *Distribution of lymphocyte subpopulations in the blood of patients with active rheumatoid arthritis (RA) and normal controls*

	Mean percentage ± SD		
	T cells by cytotoxicity*	E rosetting cells	B cells
RA (n=17)	80 ± 11	60 ± 7	18 ± 5
Normals (n=20)	77 ± 10	64 ± 6	18 ± 4

*Percent T cells was corrected for monocyte contamination.

from 17 patients with RA. Similar preincubations with normal AB sera were done in parallel. The cells were washed in serum-free medium after the preincubation and then cultured with PHA. Effective inhibition of lymphocyte reactivity to PHA was observed after preincubation with active RA sera at both temperatures in 34 out of 43 assays. No inhibition was demonstrated by similar preincubation with normal AB sera (Table 3).

The response of isolated E rosette forming cells could also be blocked by RA sera as found in all 6 patients and 2 normal controls that were tested on 2 different occasions (Table 4).

Table 3 *Blocking PHA responsiveness by sera from patients with active rheumatoid arthritis (RA). Relative responsiveness of normal peripheral blood lymphocytes to PHA after 30 minutes preincubation with 50% serum (v/v) from patients with active rheumatoid arthritis (RA) and normal AB controls is compared to the response of the same cells in serum-free medium*

Preincubation temperature	Mean % of normal PHA responsiveness in serum-free medium ± SD	
	Preincubation in normal AB sera	Preincubation in active RA sera
	n=43	n=43
4°C	96.0 ± 8.5	21.6 ± 13.1
37°C	96.8 ± 13.9	30.1 ± 20.5

Table 4 *Inhibition of ³H-leucine uptake of unseparated and isolated E rosette forming cells by serum obtained from a patient with active rheumatoid arthritis (RA). One out of 2 experiments is shown*

		³ H-leucine uptake ± SD*	
		No serum	RA serum
Normal	Unseparated cells	46 660 ± 1760	24 960 ± 4306
	E-rosette forming cells	68 901 ± 5268	24 660 ± 1131
RA	Unseparated cells	25 360 ± 760	20 640 ± 1601
	E-rosette forming cells	37 115 ± 1424	12 760 ± 3400
RA	Unseparated cells	26 170 ± 2762	21 480 ± 1093
	E-rosette forming cells	28 375 ± 1581	14 785 ± 567
RA	Unseparated cells	40 100 ± 3844	29 990 ± 5594
	E-rosette forming cells	48 253 ± 2500	24 840 ± 3700

*At PHA concentration of 5.0 µg/ml.

Discussion

In-vitro PHA responsiveness of peripheral blood lymphocytes of patients with active RA and normal controls was studied by a short-term assay that yields highly reproducible results and does not require serum. Diminished PHA responsiveness of unseparated lymphocytes from patients with active RA was a frequent finding, but isolated T cells showed normal in-vitro PHA reactivity. Normal numbers of T cells (E rosette forming cells as well as T cells identified by a specific anti-T cell antibody) were found in patients with RA, indicating that T cell number and function were grossly unimpaired in RA. On the other hand we found serum factors that block normal lymphocyte proliferative responsiveness to PHA in patients with active disease. Efficient blocking followed 30 minutes' interaction of RA sera and normal lymphocytes. Isolated T cells from RA patients that showed a normal PHA reactivity when cultured in the absence of serum could also be blocked by RA sera (Table 4). Taken together the data suggest that the defect in the cell mediated responses in RA may be due to serum

blocking factors. It is possible that different lymphocyte separation procedures and culturing techniques are some of the factors that determine the degree of autologous serum contamination in the culture mixture, thus accounting for the conflicting results reported.^{3 7-9 11 12}

Our data may be the basis for some of the recent observations on the immunological status of patients with RA. Increased lymphocyte responsiveness to PHA was observed in patients at the end of intensive plasmapheresis (20 times over 11-week period) without any change in T and B cell counts (95% ³H-TdR over 66% ³H-TdR of normal donor control). The enhanced lymphocyte responsiveness was transitory, indicating that it was directly related to removal of blocking factors from the serum.¹³ Likewise sera from patients with active RA added to normal cells reproduced the depressed monocyte cytotoxicity that was observed in patients with active RA.¹² Serum blocking factors present in RA may alter the immune regulation and consequently may play a role in maintaining disease activity in RA.

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