Lymphocyte reactivity in rheumatoid arthritis

Mixed lymphocyte culture

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Caperton, E. M., Jr., Baker, D. R., and King, R. A. (1975). *Annals of the Rheumatic Diseases*, 34, 231–234. **Lymphocyte reactivity in rheumatoid arthritis. Mixed lymphocyte culture.** Lymphocyte function in rheumatoid arthritis has been assessed by the mixed lymphocyte culture assay. In contrast to previous studies, no difference was found between lymphocytes from healthy volunteers and patients with rheumatoid arthritis. An occasional patient's lymphocytes failed to respond on a given day, but were normal on restudy. All 44 arthritic patient's lymphocytes underwent blast transformation on stimulation with lymphocytes from unrelated donors. By reaction in mixed lymphocyte culture, lymphocytes from patients with rheumatoid arthritis appear to be normal and no different from normal cells studied simultaneously.

As cellular mechanisms of immune protection and injury become better defined, the role of the lymphocyte in diseases of presumed immunological origin comes under close scrutiny (Messner, 1974). Rheumatoid arthritis (RA) is an example of a chronic disease where continuous immunological activity has been shown (Fudenberg and Kunkel, 1961), and which is characterized pathologically by striking lymphocytic infiltration into the diseased, hypertrophied synovium. Studies of the lymphocytes from patients with RA have shown abnormalities of function. Silverman and Johnson (1974) demonstrated abnormal PHA response, and Paty, Sienknecht, Townes, Hanissian, and Masi (1974) found impaired response to antigenic stimulation *in vitro*, while Mellbye, Messner, DeBord, and Williams (1972) and Williams, DeBord, Mellbye, Messner, and Lindstrom (1973) found deficiencies in the number of T-lymphocytes in RA. These and similar findings indicate possible impairment of lymphocyte reactivity in patients with RA.

One test purported to show abnormal reactivity of lymphocytes from patients with RA is the mixed lymphocyte culture (MLC) (Astorga and Williams 1969; Hedberg, Kallen, Low, and Nilsson, 1971). We have examined this reaction exhaustively; results of 310 MLCs show the *in vitro* function of lymphocytes from patients with RA to be normal and no different from controls done simultaneously.

Methods

PATIENTS

Patients with classical RA (criteria of American Rheumatism Association) were selected at random from the outpatient clinic or inpatient hospital population. All patients had active arthritis at the time of study as defined by the presence of multiple inflamed joints. Therapy included aspirin, indomethacin, low-dose steroid (<7.5 mg prednisone), gold salts, and chloroquine. No patients on cytotoxic drugs were included. MLC was not affected by any single drug or combination of drugs. Normal lymphocytes were donated by healthy volunteers.

BLOOD

30–40 ml venous blood was drawn into plastic syringes containing approximately 1000 units sodium heparin.

CELL SEPARATION

Blood was mixed 1:1 with Hepes buffered RPMI 1640 culture media, pH 7.25–7.35, containing penicillin 4000 units, and streptomycin 4000 µg/100 ml, L-glutamine 1.6 mmol/l, and sodium heparin 2000 units/100 ml at room temperature.

The blood-RPMI mixture was layered on Ficoll-Hypaque in 10 ml aliquots in sterile glass tubes, the Ficoll-Hypaque made by mixing 10 parts 33.9% Hypaque-M with 24 parts 9% Ficoll, final density 1.077–1.080. The tubes were centrifuged at 850 × g for 30 min at room temperature. The white cell layer was aspirated with a pasteur pipette and placed in a sterile glass tube containing 2–3 ml of the RPMI media—5% pooled normal human serum. The cells were washed by gentle aspiration and centrifugation at 850 g three times with fresh media and finally suspended in 6 ml of the RPMI media—15% serum. 2–3 ml of this cell suspension was placed in a sterile glass tube,

brought to a final volume of 6 ml by the addition of the RPMI media—15% serum, and mixed with 0.6 ml of mitomycin C (300 µg). The mixture was incubated at 37°C in a waterbath for 30 min after which the cells were washed three times with the RPMI media—5% serum and finally suspended in 2–3 ml of the RPMI media—15% serum. Lymphocytes were counted in a haemocytometer after mixing 1 part of the cell suspension with 20 parts methyl violet—10% acetic acid. Lymphocytes were brought to a final concentration of 2×10^5 cells/ml by mixing with the RPMI media containing 15% pooled normal human serum from male donors with red blood cell type AB+.

MIXED LYMPHOCYTE CULTURE

One-way MLC were set up by mixing 10^5 untreated lymphocytes (0.5 ml) with 10^5 mitomycin-C treated lymphocytes (0.5 ml), and cultured in 2 ml polypropylene tubes at 37°C in a humidified 5% CO₂ incubator for 96 hours. In some experiments, cells were also cultured for 160 hours. At 96 hours (or 160 h), 50 µl thymidine-2-¹⁴C, specific activity, 50 µCi/50 ml, was added to each tube and the culture continued for 24 hours. The cultures were stopped by refrigeration at 4°C.

Cells were harvested by passing the complete culture suspension through a millipore filter holder containing Whatman glass fibre paper. The culture tubes were washed twice with water to ensure complete harvest of all cells, the wash passed through the same filter paper. The cell-containing filter paper was placed in a polyethylene counting vial, 4 ml of Instagel scintillation fluid added, and the sample counted for 1 min at room temperature. All experiments including background controls (unstimulated) were done in triplicate and results recorded as mean of the three cultures.

The amount of blast transformation was calculated as a Stimulation Index (SI):

$$SI \ L_1 \times L_{2m} = \frac{\text{counts/min } L_1 \times L_{2m}}{\text{counts/min } L_1 \times L_{1m}}$$

where L_1 = lymphocytes from patient 1, and L_2 = lymphocytes from patient 2, m indicating mitomycin-treated cells. An SI > 3 was arbitrarily selected as representing significant blast transformation. Statistical analysis employed χ^2 with Yates's correction.

Results

A total of 310 mixed lymphocyte cultures (MLC) were done, using 44 patients with RA and seventeen normal control volunteers (NC). Of the 310 MLCs, 276 were 96-h and 34 were 160-h cultures. The results are shown in Table I. There is no statistical difference between any of the groups. Comparing the RA by RA group to the control by control group, $\chi^2 = 2.53$ for the entire group ($P > 0.10$), while in the 96-h cultures, $\chi^2 = 1.905$ ($P > 0.1$).

We occasionally encountered an RA patient whose cells failed as either responder, stimulator, or both on a given day. Of the 145 MLCs done in which lymphocytes from RA patients were stimulated with cells from RA patients, 17 cultures failed to undergo blast transformation ($SI < 3$). These 17 cultures involved only twelve RA patients as responder and as stimu-

Table I *Mixed lymphocyte culture in RA patients (RA) and normals (NC)*

Responding cell	Stimulating cell*	No.	Stimulation index			
			3	5	10	20
96-hour culture						
RA	RA	123	107	83	43	14
			87%	67.5%	35%	11%
RA	NC	53	45	34	19	11
			85%	64%	36%	21%
NC	RA	53	51	39	17	3
			96%	74%	32%	6%
NC	NC	47	45	39	19	6
			96%	83%	40%	13%
160-hour culture						
RA	RA	22	21	19	16	8
			96%	86%	73%	36%
NC	NC	12	12	11	8	2
			100%	92%	67%	17%
Total						
RA	RA	145	128	102	59	22
			88%	70%	41%	15%
RA	NC	53	45	34	19	11
NC	RA	53	51	39	17	3
NC	NC	47	45	38	23	5
			96%	81%	49%	11%
Total		310	281	225	122	94
			91%	73%	39%	14%

*Stimulating cell—mitomycin treated.

Table II *Variability of MLC of one patient (BS) studied twice*

Responding cell	Stimulating cell	Date	Stimulation index
BS	LOD	19 Oct 73	1.4
BS	AD	19 Oct 73	2.2
BS	CM	6 Dec 73	9.3
BS	OH	6 Dec 73	7.2
BS	LA	6 Dec 73	6.5

All patients have RA.

lator, 9 of the cultures involving three patients. Without exception, all patients whose cells failed to stimulate or respond on one occasion were restudied, and all responded and stimulated with significant blast transformation on restudy. Table II shows results of repeat study of one such patient, indicating that when first studied his cells failed to respond, whereas 6 weeks later they responded normally to cells from three RA patients. Similar data were obtained from all patients who failed to respond on one study so that all 44 patients showed normal response to heterologous lymphocytes derived from patients with RA.

Discussion

Rheumatoid arthritis remains a disease of unknown aetiology, although there is now considerable evidence

to suggest an altered immunological system being important in its development and chronicity. Morphologically the articular lesion in RA is characterized by a dense infiltrate of lymphocytes, with small nodular aggregates, the Allison-Ghormley nodules. Serological changes found in RA include the presence of rheumatoid factors (Fudenberg and Kunkel, 1961), antinuclear antibodies (Pollak, 1964), LE cells (Friedman, Sickley, Poske, Black, Bronsky, Hartz, Feldhake, Reeder, and Katz, 1957), and an altered synovial fluid complement in the face of a normal serum complement level (Bunch, Hunder, Offord, and McDuffie, 1974; Ruddy and Austen, 1970). These serological and morphological changes appear to be the immunological response to an unknown stimulus, but do not allow differentiation between a normal and an abnormal response.

Several recent studies suggest that the lymphocyte itself is abnormal in RA. Using one-way mixed lymphocyte cultures (MLC) as a means of investigating the normalcy of lymphocytes in RA, Astorga and Williams (1969) found that in 14 of 22 matches there was no lymphocyte stimulation when RA lymphocytes from unrelated donors were mixed. Hedberg and others (1971) found a similar failure of blast transformation in 3 of 10 matches of lymphocytes from unrelated donors with RA.

Our initial data failed to show significant differences between normal and RA cells in MLC (Caperton, Baker, and King, 1974), while Metzger, Opelz, Bluestone, and Terasaki (1974) recently reported normal MLC response from 34 patients with RA.

The response to MLC has now been shown to be controlled by a locus (or loci) in the major histocompatibility region, with the MLC locus closely linked to, but separated from, the loci controlling the HL-A antigens (Amos and Yunis, 1971). The HL-A and the MLC loci are polymorphic, each containing a large number of alleles (Albert, Mempel, and Grosse-Wilde, 1973). As such, it is unlikely that two unrelated individuals will be MLC identical, and studies in the random unrelated population show about 0.1% nonstimulation. Since the significance and genetics of the MLC response have only recently been defined, we felt it was important to investigate the nature of the lymphocyte reactivity in RA and to extend our initial study of MLC.

We found that peripheral blood lymphocytes from patients with RA responded normally in MLC when compared with normal cells done simultaneously (Table I). A total of 310 MLCs were done involving 44 patients with RA and seventeen normal controls. There were 17 of 145 RA by RA reactions involving twelve RA patients whose cells either failed to stimulate or to respond with significant blast transformation in initial study. All twelve patients who failed to respond or stimulate even a single responder were

restudied 2 to 6 weeks later. On restudy, cells from all patients with RA stimulated and responded to RA cells, resulting in significant blast transformation. Table II shows results of one such study.

While there is no statistical difference in MLC between RA patients and normal controls done simultaneously in this study, there were on initial study twelve of 44 rheumatoid patients whose cells either failed to respond or failed to stimulate cells from at least one RA patient (though 9 of the 12 did stimulate and respond to cells from other RA patients in the same experiment). That lymphocytes may differ from day to day in their reactivity is well known. Infection with rubella (Olson, Dent, Rawls, South, Montgomery, Melnick, and Good, 1968), ingestion of English walnuts (Marquardt, Snyderman, and Oppenheim, 1973), and the presence of cytotoxic or blocking antibodies with anti-HL-A activity in the sera (Ceppellini, Bonnard, Coppo, Miggiano, Pospisil, Curtoni, and Pellegrino, 1971) have all been reported to interfere with lymphocyte reactivity. One may speculate that a host of such factors may alter lymphocyte activity. This offers one explanation for the poor response observed in 17 of 145 cultures in this study, and in 17 of 32 of the combined results of Astorga and Williams (1969) and Hedberg and others (1971).

The technique employed in the present study differs in two important ways from that of the previous studies, which may better explain the differences in results. The cell separation technique employed by Astorga and Williams yielded a cell suspension containing 22-30% lymphocytes, whereas our separation on Ficoll-Hypaque yielded cell suspensions of 90-95% lymphocytes. Secondly, our cultures were prepared and maintained in RPMI media containing normal male human serum, while Astorga and Williams used Hanks's solution to wash and MEM-containing fetal calf serum (FCS) to culture the cells.

We tested the possibility that the differences may be related to the use of FCS rather than human serum (HS) in 24 experiments. All 24 MLCs with HS in the media underwent significant blast transformation, with 22 of 24 having $SI > 5$. In cultures done simultaneously with FCS in the media, the SI was >3 in only 16 of 24 and >5 in only 7 of 24 cultures. There was great variation in batches of FCS, so that all cultures may respond in one batch while less than half respond in another.

Further studies of lymphocytes in RA are important and are underway. This study fails to answer the basic question of whether the lymphocyte in patients with RA is normal, but the MLC response appears to be no different than that of normal controls.

Supported by a grant from the Minnesota Division, Arthritis Foundation.

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