

Acta Medica Okayama

Volume 32, Issue 6

1978

Article 8

DECEMBER 1978

Low responsiveness of synovial and peripheral blood lymphocytes stimulated by either PHA-P or CON-A in patients with chronic rheumatoid arthritis

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Abstract

Lymphocytes were highly purified from synovial fluid and peripheral blood of 10 rheumatoid arthritis patients and assessed for responsiveness to PHA-P and Con-A. In all cases, both synovial and blood lymphocytes showed a marked reduction in response to these mitogens compared with normal blood lymphocytes. The factors responsible for this low T cell responsiveness are discussed.

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Acta Med. Okayama 32, (6), 441—447 (1978)

**LOW RESPONSIVENESS OF SYNOVIAL AND PERIPHERAL
BLOOD LYMPHOCYTES STIMULATED BY EITHER
PHA-P OR CON-A IN PATIENTS WITH CHRONIC
RHEUMATOID ARTHRITIS**

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Received August 11, 1978

Abstract. Lymphocytes were highly purified from synovial fluid and peripheral blood of 10 rheumatoid arthritis patients and assessed for responsiveness to PHA-P and Con-A. In all cases, both synovial and blood lymphocytes showed a marked reduction in response to these mitogens compared with normal blood lymphocytes. The factors responsible for this low T cell responsiveness are discussed.

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Clinical and laboratory findings suggested a relationship between cell-mediated immunity and joint lesions in chronic rheumatoid arthritis (RA) (1-3). To investigate the role of cell-mediated tissue damage in RA, the responsiveness of blood lymphocytes to nonspecific mitogens such as PHA and Con-A or to possible specific antigens such as denatured IgG and synovial tissue extracts has been studied by a large number of workers. However, no consistent results have been obtained: *e.g.* the response was reported normal by Kacaki *et al.* (4), Waxman *et al.* (5), and Menard *et al.* (6); increased by Ivanyi *et al.* (7); and decreased by Griswold and McIntosh (8), Reynolds and Abdou (9), Lance and Knight (10), and Rawson *et al.* (11). The findings remain controversial.

In contrast, there have only been a few reports of work using lymphocytes taken directly from the synovial cavity of damaged sites. Ivanyi *et al.* (7), Reynolds and Abdou (9), and Sheldon *et al.* (12) reported a decreased response to PHA. Our experience with synovial fluid of RA patients has revealed that the cytological picture differs widely among cases. In addition, the Hypaque-Ficoll separation technique used in previous studies is not sufficient to purify the lymphocytes.

In this paper, therefore, we used highly purified lymphocytes from synovial

fluid and tested the responsiveness of a constant number of lymphocytes to PHA and Con-A. Low responsiveness was confirmed for both synovial fluid and blood lymphocytes. In a following paper, the screening of environmental factors which might cause this decreased response to nonspecific mitogens will be reported.

PATIENTS AND METHODS

Patients. Seventeen patients with definite and classical RA were studied. Only 10 cases yielded sufficient numbers of purified synovial lymphocytes (more than 5×10^5 cells) for testing blastogenesis. All patients were under medication, mainly oral administration of small amounts of prednisolone (less than 10 mg a day) and non-steroid hormones. Some patients had been receiving intra-articular administration of steroid hormones which was stopped at least 2 weeks before collection of synovial fluid.

Purification Procedures. Synovial fluid was collected in the presence of hyaluronidase, 5 units/ml and sodium heparin, 250 units/ml. This was then diluted 3-fold in Hanks' BSS (balanced salt solution), and Hypaque-Ficoll centrifugation was performed in the usual manner to remove the majority of the highly contaminating polymorphonuclear cells. Cells at the interface were suspended in Tissue Culture Medium-199 containing 10% (v/v) foetal calf serum (FCS) and incubated in a glass Petri-dish for 40 min at 37°C twice to remove adherent cells. After incubation, loosely adherent lymphocytes were recovered by agitation with pipetting. The differential cell count for the resultant lymphocyte-rich cell suspension was checked on cytocentrifuge preparations stained with May-Giemsa or Peroxidase. Peripheral blood lymphocytes were separated on Hypaque-Ficoll. All procedures were performed under sterile conditions.

Blastogenesis. Lymphoid cell suspensions were adjusted to exactly 5×10^5 cells/ml in TC-199 containing 10% FCS. Aliquots of 200 μ l were added to the wells of a Microplate II tissue culture plate. PHA-P (Bacto-phytohemagglutinin P, Difco), 3.0 μ g or Con-A (Sigma Co.), 2.0 μ g was then added. The concentration of mitogens used was determined from a dose-response curve which included 0.3, 3.0 and 30 μ g for PHA-P and 0.2, 2.0 and 20 μ g for Con-A. Experiments were performed in triplicate for each preparation. Incubation was continued for 72 h in a CO₂ incubator and 4 h before cell harvesting, 1 μ Ci of ³H-thymidine was added in each well. A Mini-MASH automatic cell harvester was used to collect and wash the cells and radioactivity as counts per minute (c.p.m) was counted in a Liquid Scintillator. Normal peripheral blood lymphocytes were used as controls under exactly the same conditions.

T and B cell counts. T cells were counted by spontaneous sheep red blood cell (SRBC) rosette formation, and B cells by the presence of surface immunoglobulins (S-Ig). For detecting S-Ig, goat anti-human heavy chain (γ , α , μ -chain) monospecific antibody labelled with FITC (Hyland) was used. Briefly, after the purification procedure, approximately 1×10^5 cells were incubated with 1 drop of conjugate (centrifuged at 10,000g before use) at room temperature for 30 min,

followed by 3 washes in PBS containing 0.02% (w/v) NaN₃. Stained cells were mounted on a glass slide in phosphate-buffered glycerol and observed by conventional fluorescent microscopy.

RESULTS

Figure 1 shows the cell type distribution after purification. Larger closed circles indicate the cases used in this experiment. Smaller circles are the cases which could not supply sufficient cells for testing blastogenesis. Six out of ten cases yielded more than 80% mononuclear cells; other cases still contained large numbers of neutrophils and macrophages even after purification. These results were supported by peroxidase staining (lower column).

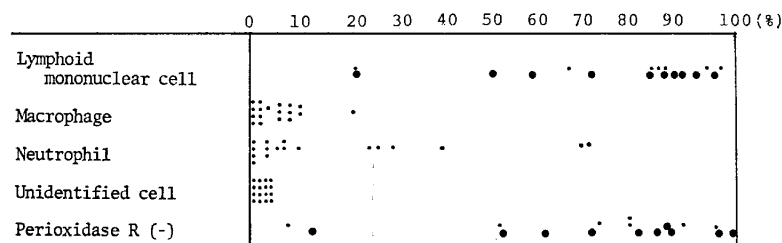


Fig. 1. Proportion of lymphoid mononuclear cells from synovial fluid of 17 RA patients purified by Hypaque-Ficoll sedimentation and double absorption to a glass Petri-dish, larger closed circles indicate cases used in this experiment.

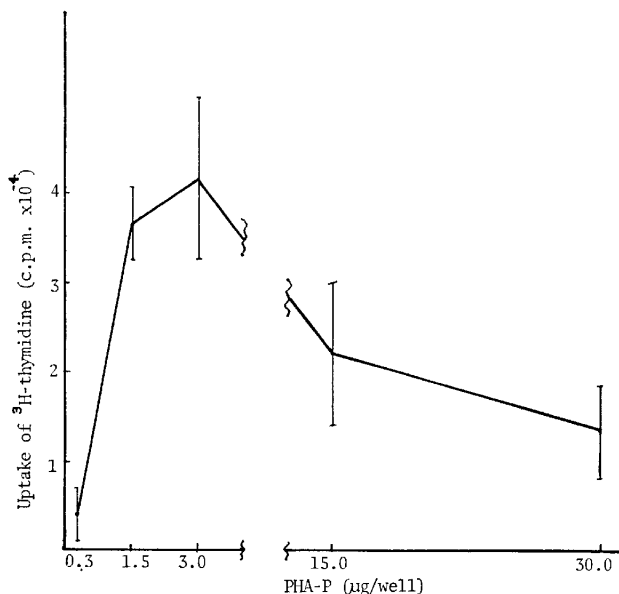


Fig. 2. Dose-response curve for PHA-P. Peripheral blood lymphocytes, 1 x 10⁵ cells in each well were incubated with 1 μCi ³H-thymidine.

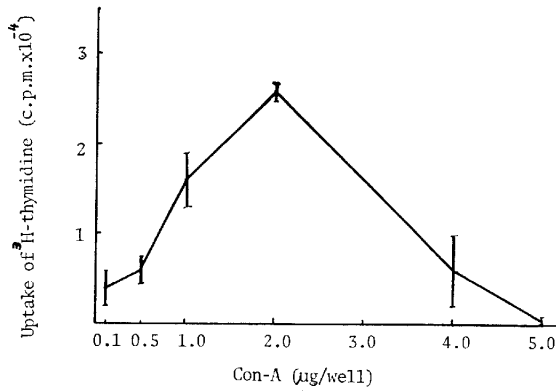


Fig. 3. Dose-response curve for Con-A. Peripheral blood lymphocytes, 1×10^5 cells in each well were incubated with $1 \mu\text{Ci } ^3\text{H-thymidine}$.

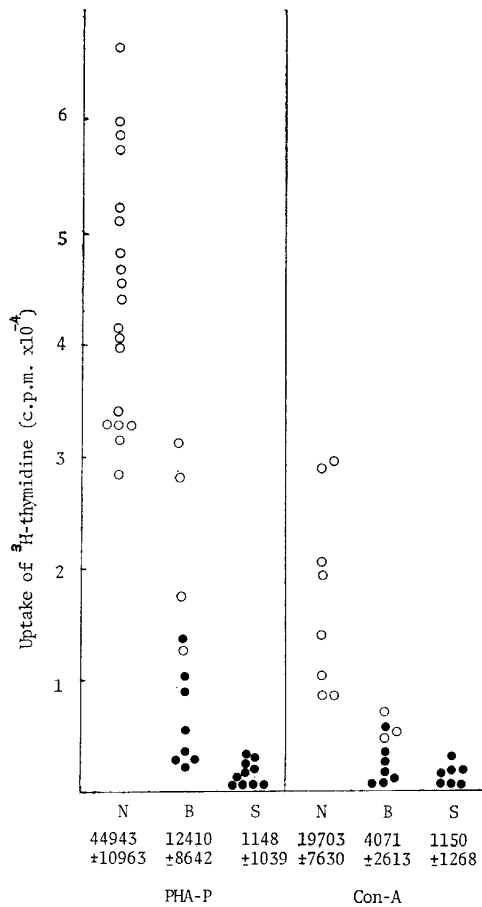


Fig. 4. Blastogenesis of synovial fluid and peripheral blood lymphocytes to PHA-P and Con-A. N, normal peripheral blood lymphocytes; B, blood lymphocytes of RA; S, synovial fluid lymphocytes of RA; open circle (○), before adhesion to glass dish; closed circle (●); after adhesion to glass dish.

In Figs. 2 and 3, dose-response curves for PHA-P and Con-A are given. PHA-P showed maximal response at a concentration of $3.0\mu\text{g}/1 \times 10^5$ cells and Con-A at $20\mu\text{g}/1 \times 10^5$ cells.

Fig. 4 summarizes the results. Blood and synovial lymphocytes showed markedly low responsiveness to both PHA-P and Con-A; approximately ten-fold for synovial lymphocytes compared with that of control blood lymphocytes.

Table 1 shows the data for each case. The proportion of T cells in synovial fluid correlated well with response to mitogens except for case No. 3. B cell proportions varied from 4.3% to 63.7% and did not correlate with mitogen responsiveness.

TABLE I. BLASTOGENESIS, CELL DIFFERENTIATION AND T, B CELL SUBPOPULATIONS IN SYNOVIAL FLUID OF RA PATIENTS

Patients	Class	Stage	Blastogenesis ^a		Mononuclear lymphoid cells (%)	Perioxidase reaction(+) (%)	T cell (%)	B cell (%)
			PHA (3.0)	Con-A (2.0)				
1 Y. M.	III	IV	1086	1655	92.0	7.9	35.0	17.8
2 S. T.	II	IV	1498	—	90.8	14.6	40.6	11.6
3 M. K.	III	IV	228	37	50.0	44.6	41.0	37.4
4 Y. U.	II	I	157	141	71.9	28.1	—	—
5 T. C.	II	III	682	—	95.7	3.6	—	—
6 M. O.	II	IV	2854	1662	—	—	—	—
7 T. Y.	II	III	1939	897	56.4	36.2	61.8	26.2
8 H. S.	I	II	2634	3543	89.9	9.0	53.0	4.3
9 Y. S.	III	IV	250	112	21.0	85.1	14.8	67.7
10 K. S.	I	IV	151	—	97.0	0	—	—

^a Blastogenesis was measured as described in methods.

Data are expressed as c.p.m. of ³H-thymidine incorporated.

DISCUSSION

Chronic rheumatoid arthritis is characterized by the infiltration of lymphoid mononuclear cells in the synovial tissue which might cause joint destruction (1, 3). Functional analysis of these cells has been carried out by many workers. In the present study, the biological activities of lymphocytes from synovial fluid were assessed by the incorporation of ³H-thymidine after stimulation with non-specific T cell mitogens. For this, it was necessary to purify lymphocyte preparations from contaminating macrophages and neutrophils. In many previously reported studies, the Hypaque-Ficoll sedimentation technique alone was used. This technique, however, is not sufficient to remove the large contamination with macrophages and even neutrophils found in synovial fluid. Further adhesion to glass Petri-dish was required to remove these cells. Even though this purifica-

tion technique was applied to synovial fluid in this study, some cases *e.g.* case No. 9 still showed a low proportion of lymphocytes. Therefore, in the present experiment, the cell concentration was adjusted to give a constant total number of lymphocytes for the blastogenesis to mitogens. Early death of contaminating macrophages and neutrophils during the 72 h incubation did not influence the results.

Ivanyi *et al.* (7) reported an increased response of RA synovial lymphocytes to B cell mitogens such as PWM and LPS, but a decreased response to T cell mitogens such as PHA. Sheldon *et al.* (12) also reported a decreased response to PHA, approximately one quarter of that of normal blood lymphocytes. Our data showed a response approximately one tenth of normal. The average response for triplicate experiments varied case by case. Lymphoid cell suspensions for cases No. 5 and 10 contained more than 95% lymphocytes before incubation and showed a markedly low responsiveness. It is not clear whether the response was really low or was reduced by the lack of macrophages in these particular preparations (13). Blood lymphocytes also showed a reduced response to PHA and Con-A. Our data confirm the results of previous studies (9, 11, 12, 14).

Why do lymphocytes in RA show a low response to mitogens? Several explanations can be considered. Low complement levels and the presence of rheumatoid factor in the synovial fluid have been reported. These findings suggest the presence of immune complexes which might cause a lowered responsiveness to mitogens. Large amounts of synovial tissue antigens and denatured IgG are also present in the synovial fluid which could have already stimulated the lymphocytes *in vivo*, thus resulting in poor *in vitro* blastogenesis to nonspecific mitogens. In addition, therapeutic drugs might cause a lowered response to mitogens. In this experiment, an attempt was made to avoid such an effect by ceasing the administration of drugs at least two weeks before collection of the test samples. Reduced biological function of synovial lymphocytes does not deny their role in synovial tissue destruction. In fact, Stastny *et al.* (2) reported lymphokine activity in synovial fluid. The lymphocytes, therefore, might have lost their biological activity *in vivo* after releasing lymphokines. Data on blood lymphocytes in RA, however, might suggest the presence of humoral factors which directly cause a lowered responsiveness.

Reported T and B lymphocyte proportions in synovial fluid vary widely (15, 16). Sheldon *et al.* (12) found increased T cell and decreased B cell proportions. In contrast, Venon-Robert *et al.* (17) reported decreased T cells and increased B cells. This discrepancy may be explained by differences in the purity of test lymphocyte suspensions, the nature of the conjugated anti-immunoglobulin used or by the presence of exogenous cytophilic immunoglobulin on the cell surface. Winchester *et al.* (19) recommended the use of conjugated anti-F (ab')₂ to avoid

binding via the Fc receptor. This is, however, still controversial. To remove the majority of exogenous immunoglobulin in this study, lymphocyte suspensions were incubated at 37°C for 1 h and washed before adding the conjugate.

In a subsequent paper, humoral factors in the synovial fluid which might affect lymphocytes will be examined.

Acknowledgment. We wish to thank Dr. J. M. Rolland, Department of Pathology and Immunology, Alfred Hospital, Prahran, Victoria, Australia for scientific discussion and assistance with the completion of this manuscript.

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