Characteristics of spontaneously proliferating mononuclear cells in rheumatoid arthritis

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SUMMARY The phenomenon of increased spontaneous incorporation of ³H-thymidine (³H-TdR) into peripheral blood mononuclear cells in rheumatoid arthritis (RA) has been investigated. The activity was found to be short lived and affected less than 1% of cells. Using a Percoll density gradient we identified two populations of active cells. RA patients with active synovitis and increased ³H-TdR incorporation in the low density population of cells have higher overall ³H-TdR incorporation than normal controls and patients with inactive RA. The low density cell population is enriched for Ia⁺ cells. The data are consistent with raised spontaneous ³H-TdR incorporation being due to an in-vivo cell mediated immune response.

Key-words: immunity, cellular, lymphocyte activation, Ia antigen, autoimmune disease.

The nature of the events which initiate, and then allow, the persistence of inflammation and immunological abnormalities in rheumatoid arthritis (RA) is poorly understood. 'Atypical' lymphocytes have been described in RA¹ but the questions of the lineage of these cells and whether they are the same as those described in infectious mononucleosis and renal transplantation have not been answered conclusively.^{2–7}

Developments in monoclonal antibody technology and methods for studying the interactions between lymphocyte subpopulations have led to a renewed interest in in-vivo activated lymphocytes in a variety of autoimmune diseases, including RA.^{8–10}

We have approached this question in two ways. We have used the phenomenon of spontaneous incorporation of ³H-TdR into mononuclear cells as the criterion of activation, and have asked firstly whether the spontaneous activity seen in peripheral blood mononuclear cells from RA patients resembles the proliferative phase of a mitogen or alloantigen stimulated lymphocyte reaction, and secondly whether the activity relates to disease status. In this first report we describe the occurrence

of spontaneous activity with time in peripheral blood mononuclear cells of RA patients and its relation to other disease parameters. We also present the results of preliminary investigations into the nature of the activated cell.

Materials and methods

Subjects. The patients selected had active RA¹¹ but were not receiving penicillamine-like drugs or steroids; they had not had recent surgery, a blood transfusion, or infection. Controls were healthy members of staff, age and sex matched with the patients, and tested concurrently with the patients. Blood samples were always taken between 8 and 10 am to minimise any possible diurnal effects. In the longitudinal study, samples from 12 patients and 10 controls were tested serially over a period of four to eight weeks each, with intervals of two to seven days between samples. For the density gradient experiments samples from 23 RA patients, 16 of whom were receiving either oral or injectable gold therapy, were tested. The patients were assessed clinically at the time of blood sampling by means of a multiparameter assessment devised by Mallya and Mace.¹² In addition serum immunoglobulins, complement components, C-reactive protein (CRP), erythrocyte superoxide dismutase activity (SOD), haemolysate

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thiol (LSH), and acute phase proteins were measured.¹³⁻¹⁷ (Shapiro D, paper in preparation). Six patients and four of the 13 controls were tested on two occasions. Details of the patients and controls are given in Table 1.

Lymphocyte cultures. For the longitudinal study spontaneous ³H-TdR incorporation was measured in whole blood cultures. 5 ml of venous blood was added to 5 ml of RPMI 1640 medium containing ³H-TdR (10 µCi, specific activity 0.2 Ci/mM, Radiochemicals, Amersham) in loosely stoppered plastic tissue culture tubes. The tubes were incubated at 37°C in a humid 5% CO₂ incubator for 4 hours, after which the non-adherent mononuclear cells were separated by Ficoll-Hypaque.¹⁸ The ³H-TdR incorporation into 5 replicates of 2 × 10⁵ cells was measured with a Titertek automatic harvester and a scintillation counter (LKB). Before being counted the filter discs containing the ³H-TdR were immersed for 15 minutes each in two changes of 5% trichloroacetic acid and two changes of methanol. This had the effect of reducing the level of background counts and the variation between replicate samples.

For the density gradient experiments mononuclear cells were separated from whole blood by Ficoll-Hypaque and resuspended at 2 × 10⁶ cells/ml in RPMI 1640 containing 20% pooled male human serum (PHS) and ³H-TdR (1 µCi/ml sp.act. 50 Ci/mM). 2 ml aliquots were incubated in the flat-bottomed wells of Linbro multiwell plates (Flow Laboratories, Irvine) in a 5% CO₂ incubator. After 18 hours the non-adherent cells were washed and resuspended in RPMI 1640/10% FCS at 10 × 10⁶/ml for the activity gradient or 1 × 10⁶/ml for measuring spontaneous ³H-TdR incorporation. The spontaneous incorporation of ³H-TdR into 2 × 10⁵ cells was measured as described above.

Autoradiography. Slides were made from each sample of washed, radioactive cells with use of a cytocentrifuge. An aliquot of 0.1 ml of cell suspension (containing 1 × 10⁵ cells) was used for each slide, centrifuging at 900 rpm for 5 minutes. The slides were air dried and fixed in ethanol for 10

minutes. They were then dipped in Ilford L-4 emulsion diluted 1:2 with distilled water, held vertically in a stream of cool air to dry, and exposed for 14 days at 4°C in a light-proof desiccated box. The slides were developed for 5 minutes in Kodak D-19 developer, rinsed, fixed for 5 minutes in Amfix fixative diluted in 1:4 with distilled water, and washed for at least 2 minutes in running tap water. The cells were then stained with Giesma. One thousand mononuclear cells were scored to determine the percentage which had taken up ³H-TdR.

Activity profile using 'Percoll' density gradients Isotonic Percoll (Pharmacia) was mixed with MEM/10% FCS to give a final density of 1.060. Batches of polycarbonate tubes (MSE) containing 10 ml of diluted Percoll were ultracentrifuged at 20 000 g for 15 minutes at 4°C in a 24½° angle headed rotor. 0.5 ml of cell suspension (5 × 10⁶ cells) or 0.5 ml of a suspension of density marker beads were layered on to gradients from the same batch and centrifuged for 30 minutes at 400 g. With a red hot 21 G needle a hole was burned in the bottom of the tubes containing cells, and the gradient was collected in 0.5 ml fractions. The ³H-TdR activity was measured as described above in triplicate 100 µl aliquots taken from each fraction. A density curve was drawn by measuring the position in the gradient of each band of coloured beads of known density. Total lymphocyte activity was computed by summing the area under the whole density gradient curve, and the activity of the two subpopulations was expressed as the area under each activity peak.

Membrane staining. Monoclonal antibody (Orthomune OKIa1 or OKM1) was added at a 1:40 dilution to 10⁶ washed mononuclear cells in 0.2 ml RPMI 1640/10% FCS and the tubes incubated in the dark for 30 minutes at 4°C. The cells were washed three times, resuspended in a 1:10 dilution of FITC conjugated rabbit anti-mouse IgG serum (Nordic) in RPMI 1640/50% PHS, and incubated for a further 30 minutes at 4°C. After a further three washes the cells were resuspended in 2 drops of a 1:1 PBS-glycerol mixture. One drop was put on to each of two slides, and overlaid with coverslips which were sealed with nail varnish. Two hundred cells were counted on each slide under phase contrast microscopy and incident ultraviolet light to determine the percentage of fluorescing cells.

Statistical analysis of results. Statistics were carried out with either the Minitab statistical package¹⁹ or the BMDP package,²⁰ both of which are available on the Glasgow University ICL 1900 computer. The autoradiography results from patients and controls were compared by the Mann-Whitney U test. The

Table 1 Details of patients and controls

	Longitudinal study		Density gradient study	
	Patients	Controls	Patients	Controls
No	12	10	29	17
Mean age	56	41	46	40
(range)	(37-75)	(25-56)	(22-72)	(27-58)
M/F	2/10	5/5	5/29	6/11
RF positive	11	ND	9	ND

ND=not determined.

lymphocyte activity results were transformed to give an approximately normal distribution and analysed by a 2-sample *t* test (for populations with different variances). The laboratory data were analysed by Hotelling's multivariate *t* test.

Results

Serial measurements of spontaneous incorporation of ^3H -TdR

Raised levels of spontaneous ^3H -TdR incorporation were seen in 4 out of 12 patients (33%). The increase was transient, with each of these patients showing normal results at some point during the study. There was no consistent trend up or down in the results. The data are therefore expressed as vertical lines (Fig. 1), with each line representing all the results obtained during the observation period of each individual studied and the points on each line representing the separate measurements for that individual.

Autoradiography

The median values (and interquartile ranges) of ^3H -TdR positive cells per 1000 mononuclear cells were 3(1-6) and 1(1-3) for the pooled results of patients and controls respectively. The results showed a small but significant increase in the median level of positive cells in patients compared with controls ($p < 0.001$), suggesting that the raised ^3H -TdR incorporation was due to a small number of highly active cells.

Density profile of spontaneously active cells

A typical density profile showing two peaks of activity is shown in Fig. 2. The total activity, expressed as the area under the curve, was not significantly different in the patient group as a whole compared with controls. A subpopulation of five patients (21%), however, had levels of activity which were above the normal range (Fig. 3). The results of both patients and controls could be divided into those in which there was a single peak of activity in the density range 1.06-1.08 (group I) and those in which there was an additional second peak in the density range 1.03-1.06 (group II). Eighteen of 29 (62%) of the patient results and 8/17 (47%) of the control results were categorised as group II. Active lymphocytes with a density between 1.06 and 1.08 are referred to as high density lymphocytes (HDL) and those with a density between 1.03 and 1.06 as low density lymphocytes (LDL). The patients with LDL (group II) had both a higher total activity than the controls with or without LDL (Table 2).

Clinical and laboratory assessment

Twenty-six of the 29 RA patients completed 12 weeks of follow-up, and the results of their clinical and laboratory indices are summarised in Table 3. Compared with group I the group II patients had slightly raised ESR, CRP, and LSH levels, and reduced SOD activity. A multivariate analysis of these results²⁰ showed a significant difference between the groups ($p = 0.03$). Considering change in

Fig. 1 The spontaneous incorporation of ^3H -TdR into 10^5 mononuclear cells in patients (●) and controls (○).

Fig. 2 Density profile of spontaneously activated cells.
○ = Density gradient drawn from measuring the position of density marker beads.
● = Mean ³H-TdR incorporation of aliquots.

disease status by a combination of parameters according to the method of Mallya and Mace, and the physician's overall clinical impression, we found, 8 of 11 (73%) of group I patients had improved clinically compared with 8 of 18 (44%) of group II patients.

There was an unusually high proportion of patients who were seronegative for rheumatoid factor (RF) in the patient sample as a whole, and they were equally distributed between groups I and II. However, all the patients who were seropositive (n=8) were in group II.

Morphology of activated cells

Morphological examination was carried out on the autoradiography preparations of unfractionated, non-adherent cells. Two types of ³H-TdR positive cells were seen: the first was a cell which had the appearance of a medium to large lymphocyte with an oval-shaped eccentric nucleus; the second was

similar in size but with a characteristic U-shaped or bilobed nucleus. However, the majority of large mononuclear cells were negative for ³H-TdR. The activated cells were usually very heavily labelled with isotope and were therefore easily distinguished from negative cells. Less frequently, lightly labelled cells were seen.

Surface phenotype of low and high density cells

In preliminary experiments we have attempted to recover sufficient cells from the low and high density fractions to carry out surface phenotyping. Our

Table 3 Comparison of clinical and laboratory indices of patients in groups I and II

	Group I	Group II
Age (yr)	45.6	46.1
Duration of RA (yr)	7.2	6.5
Positive for RF (%)	0	44
Clinical improvement (%)	73	44
Disease activity score	109	120
ESR (mm/h)	21	32
C-reactive protein (mg/l)	11.2	24.2
Caerulo plasmin oxidase (mg/l)	650	515
Lysate thiol (μmol/l)	384	420
Superoxide dismutase (μg/ml)	784	478
IgG (μg/ml)	109	119
IgA (μg/ml)	19.0	11.6
IgM (μg/ml)	12.3	11.6
C3 (μg/ml)	939	832
C4 (μg/ml)	333	316
CH50 (units/ml)	158	154

Table 2 Spontaneous lymphocyte activity in groups I and II

	Total activity	Activity of HDL
<i>Patients</i>		
Group I (n=11)	21.1	17.4
Group II (n=18)	44.5*	31.5*
<i>Controls</i>		
Group I (n=9)	25.5	21.6
Group II (n=8)	24.8	13.3

Results are expressed as the median area under the curve.
*Group II significantly different from group I and from normal controls.

Except for RF positivity and clinical improvement, results are expressed as the mean.
SI conversion: μg/ml=mg/l.

Fig. 3 Total spontaneous incorporation of ^3H -TdR, measured as the area under the density curve. $\circ$ =Group I results. $\bullet$ =Group II results.

results indicate a positive selection for Ia⁺ cells in the LDL of both patient and control samples. The percentage Ia⁺ cells in the LDL was increased to 34% (mean of three experiments) and in the HDL it was reduced to 10% (mean of three experiments). There was no enrichment of OKm1 (monocyte) staining cells in the LDL.

Discussion

In an attempt to clarify the nature of the immunological processes underlying RA we have examined the phenomenon of spontaneous ^3H -TdR incorporation into peripheral blood mononuclear cells in patients and in normal controls. Our longitudinal

study of 12 patients showed that 33% had raised ^3H -TdR incorporation at some point, and that the activity was transient. This finding together with the autoradiography results, showing that activity is due to a small proportion (between 0.5 and 2%) of highly active cells, is consistent with the view that ^3H -TdR incorporation represents the proliferative phase of lymphocytes undergoing a cell mediated immune response.^{21 22}

Because of the small number of cells involved, we chose a method of investigation which did not depend on depletion of particular subsets: rather, a continuous Percoll density gradient which could be reliably reproduced was used to 'fingerprint' the activity in each sample.

By this means we found that both patient and controls could be separated into those in whom activity was measurable in cells of only one density (corresponding to that of small lymphocytes)—group I—and those in whom two populations of active cells could be identified—group II. In this group, in addition to the 'high' density or small leucocyte population (HDL), there was a second 'low' density (1.03–1.06) peak of activity caused, we suggest, by low density lymphocytes (LDL).

The patients with LDL had significantly higher levels of ^3H -TdR incorporation, both overall and in the HDL subpopulation, than those without LDL or than the normal controls. Although LDL were also seen in a proportion of normal controls, there was no difference in these individuals in total activity compared with those without LDL.

Our results suggest that the patients with LDL may have more active disease. Although there was no difference between group I and II patients in terms of age, duration of illness, or the overall Mallya and Mace activity grade (Table 3), all the eight seropositive patients were in group II—seropositivity for RF being normally associated with more severe and systemic disease. Secondly, at the time of venesection, the examining physician (D.L.) assessed the patients in terms of whether they had improved, were stable, or had deteriorated clinically, and found that 73% of patients in group I had improved compared with 44% in group II. Thirdly, the ESR, CRP, SOD, and LSH levels showed a significant difference between the groups, which was consistent with the patients in group II having more active disease than those in group I. The suggestion that LDL are associated with active disease is in agreement with the findings of Papadimitriou *et al.*,²³ who, though using different criteria to define lymphocyte activity, distinguished very active and quiescent RA on the basis of low density lymphocytes.

The phenomenon of activated LDL, however, is

transient, as shown by five individuals who were tested twice and showed LDL activity on only one of the two occasions. It may be that there are subgroups of RA patients who differ in their ability to respond immunologically,²⁴ and that in-vivo activation of lymphocytes is possible only in those patients whose immune responsiveness has not been suppressed by their disease.

In samples in which sufficient cells could be recovered from the low density fraction we have shown that this subpopulation is invariably enriched for Ia-positive cells. This was shown both in patient and control samples. It would appear therefore that there is no disease specific cell associated with RA, rather that patients have both increased spontaneous proliferation in the small lymphocyte population and an increased number of low density, Ia-positive cells.

We think it unlikely that these cells are the Ia-positive dendritic cells found in rheumatoid synovial tissue,^{25 26} since the cells in our experiments were depleted of adherent cells. Although they did not stain with OK-M1 (antimonocyte) antibody, some of ³H-TdR positive cells were noted in the autoradiography preparations to have a morphological appearance resembling monocytes. Further studies on the ultrastructure of the LDL should clarify this point and are in progress.

The Ia-positive cells may be activated T cells,²⁷ the result of a cellular immune response. However, the finding that Ia-positive lymphocytes are able to present antigen^{28 29} raises the intriguing possibility that these cells might be involved in perpetuating the immune response in chronic RA patients by providing an antigenic stimulus.

In conclusion, therefore, we have shown that a proportion of RA patients have increased levels of spontaneous ³H-TdR incorporation compared with normal controls. The activity is associated with two populations of cells which can be separated by density. More active disease appears to be associated with increased ³H-TdR incorporation in a low density cell population which is characterised by an enrichment of Ia-positive cells. Further work on the morphology and the function of activated cells in both populations is required to clarify the type of immunological activity that is taking place.

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