Incorporation of ^{14}C-thymidine by cultures of erythrocytes from rheumatoid arthritis patients and normal subjects, suggesting the presence of an L-form

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Summary. Cultures of washed erythrocytes from rheumatoid arthritis patients and normal subjects were found to incorporate ^{14}C-thymidine, *suggesting the presence of an L-form.* The extent of the incorporation by erythrocytes from rheumatoid patients was more variable than that by erythrocytes from normal subjects, and correlated negatively with IgG measurements and white cell counts performed on the patients' bloods, although not with the clinical activity of the patients.

Recent cultural evidence suggests that *Bacillus licheniformis,* which has been reported as occurring in the blood and joint fluids of rheumatoids[1], also occurs as an L-form associated with the erythrocytes of normal persons[2,3]. Tedeschi and Amici[4] provided some evidence for the existence of such L-forms in the form of metabolic studies, including the uptake of thymidine into erythrocytes in culture, and subsequently demonstrated that thymidine was incorporated into DNA in suspensions of human platelets.

The work described was undertaken to investigate, with a view to possibly reinforcing the existing evidence on the occurrence of the L-form, a) whether ^{14}C-thymidine is incorporated into DNA by suspensions of erythrocytes of rheumatoid and normal subjects in culture, b) whether there is a difference in the extent of incorporation between erythrocytes from rheumatoid patients and those from normal subjects, c) whether the degree of incorporation by cultures of erythrocytes from rheumatoid patients correlates with any of the clinical tests applied to the patients, particularly those relating to disease activity.

Materials and methods. Blood was taken by syringe from 29 unselected rheumatoid patients; 21 were female and the mean age was 55 years (range 20–80 years); 8 were male and the mean age was 50 years (range 36–58 years). 16 were classical, 12 definite and 1 probable according to the ARA classification[5]. A detailed case history was compiled for each patient, with particular reference to the duration of the illness, the symptoms experienced including early morning stiffness, the number of joints involved, and the degree of deformity. Patients' assessment of the amount of pain they experienced, their walking time over a measured distance, and their grip strength measured by a mercury manometer, were recorded. From the case history, clinical examination and assessment, the patients were divided into 3 activity groups – mild, moderate and severe. The following clinical laboratory tests were performed on blood from rheumatoid patients: full blood count, E.S.R., biochemical profile including alkaline phosphatase, albumin, globulin, serum aspartate transaminase (Technicon SMA 12/60 analyzer), 5-nucleotidase[6], C-reactive protein[7], haptoglobulin[8], IgG, IgM and IgA[9], and Rose-Waaler. Control blood was taken from 13 clinically healthy persons from this laboratory and 16 carefully selected outpatients attending the Haematology Department, General Hospital, Birmingham, and suffering from noninfective conditions unrelated to arthritis, to provide a similar range of age and sex to the rheumatoid patients.

Erythrocyte suspensions prepared by centrifuging these bloods at $380 \times g$ for 5 min, washing and re-suspending to the same volume in saline, were cultured 1:10 in P.P.L.O. broth (Difco, Detroit) containing penicillin 100 IU/ml and streptomycin 0.1 mg/ml, and a final concentration of 2 μCi/ml (2-^{14}C) thymidine (Radiochemical Centre, Amersham) at 37 °C. These antibiotics were incorporated in the medium to prevent multiplication of any contaminant bacteria, since the work of Tedeschi and Amici[4,10] had indicated that the metabolism of ^{14}C-thymidine by the L-forms which occur naturally in blood is relatively unaffected by either.

Immediately after inoculation and at weekly intervals thereafter, 0.2 ml of culture was applied to a glassfibre disc which would absorb the culture and act as an inert carrier. This was allowed to dry, fixed in 5% T.C.A. and washed in 5% T.C.A., ethanol and ether[11]. After drying, toluene scintillator was added, and the disc was counted for 10 min in a Searle Analytic Mark III liquid scintillation system. Whole blood and erythrocyte suspensions were counted and the results of thymidine incorporation expressed in terms of both the equivalent volume of whole blood and of a count of 5.00×10^6 R.B.C. per mm^3. The mean and variance of the data from rheumatoid patients and from control subjects was calculated for comparison (table 1).

Strict sterile precautions were taken in the collection and handling of blood, and during cultural procedures. The donor's skin was disinfected with 70% isopropyl alcohol (Steret's injection swab, Prebbles Medical Ltd, Merseyside, England) prior to the collection of blood using sterile disposable hypodermic syringes. 5 ml samples of blood were transferred to sterile screw-capped bottles containing 0.25 ml of 0.4% (w/v) aqueous heparin, which were prepared by autoclaving at 118 °C for 10 min, and were regularly tested for sterility. All blood samples and erythrocyte suspensions were cultured separately in several cultures without antibiotics as a control. A control experiment was performed without erythrocytes using culture medium deliberately contaminated by the experimenter's fingers and preincubated for 5 h, in order to determine whether a contaminant bacterium would be likely to grow sufficiently, either in the original form or as an L-form, to produce significant ^{14}C-thymidine incorporation in the presence of the antibiotics used in the test cultures. Further controls were performed using uninoculated culture medium and concentrated platelet suspensions supplied by the Blood Transfusion Service, Birmingham. The contribution of contaminating leucocytes to incorporation by erythrocyte suspensions was assessed by comparing results for washed suspensions, suspensions in which the number of contaminating leucocytes was substantially reduced by the sedimentation of blood in dextran solution before washing, and suspensions from which the leucocytes had been removed by freezing in glycerol solution followed by washing to remove the resulting leucocyte fragments. The effect of lysing the erythrocytes by slow freezing to −20 °C and the effect of 0.5 mg/ml deoxyribonuclease (crude, from beef pancreas, Sigma Chemical Co., St. Louis, USA), with 1 mg/ml magnesium sulphate, on ^{14}C-thymidine incorporation by lysed erythrocytes was also assessed.

Results and discussion. The counts obtained were low but definitely positive, indicating that some incorporation of thymidine into DNA associated with erythrocytes was taking place. This interpretation is supported by the results of the control experiments. Control cultures of blood samples without antibiotics showed that blood collected in this way only infrequently contains low numbers of contaminants.

Those isolated from the blood samples used were exclusively *Staphylococcus epidermis* and were very sensitive to the antibiotics incorporated in the test cultures. The results of isolations of L-forms and reversions from these and similar cultures are reported elsewhere[12]. The counts obtained using both uninoculated and deliberately contaminated medium were not significant, the antibiotics present in the medium suppressing completely the growth of the contaminating bacteria *(Staphylococcus epidermidis* and *Bacillus subtilis)* and not permitting formation of viable L-forms. Concentrated platelet suspensions did not incorporate thymidine significantly in the absence of plasma, confirming the findings of Tedeschi and Amici[10], although incorporation by both platelet and erythrocyte suspensions was enhanced by resuspension in plasma. It was shown that when the number of contaminating leucocytes in the erythrocyte suspensions was substantially reduced by sedimentation in dextran, no significant reduction in ^{14}C-thymidine incorporation was observed, while the destruction of the leucocytes by freezing in glycerol solution, followed by washing to remove the fragments, actually resulted in increased incorporation (table 2). Lysis of the erythrocytes by freezing was shown to have little effect on thymidine incorporation, while incorporation by lysed erythrocyte suspensions was significantly reduced by incubation in the presence of deoxyribonuclease (table 3). These results indicate that the radioactivity is incorporated into DNA, but provide no evidence as to whether thymidine incorporation takes place within or outside the erythrocyte, in view of the uncertainty as to whether purine ribosides can penetrate the red cell membrane.

Table 1 shows that incorporation occurs in cultures of erythrocytes from both rheumatoid and normal subjects and whereas there is no significant difference between the means of the 2 sets of data, the difference between their variances is significant ($p < 0.01$). This suggests that the putative L-form exists in a more variable metabolic state in a rheumatoid population than in a comparable normal one. The results of the clinical tests applied to the rheumatoid patients were examined to determine their degree of correlation with thymidine incorporation. There was no correlation with the clinical activity of the patients, however. IgG measurements showed a significant negative correlation with thymidine incorporation after 7 to 35 days' incubation ($p < 0.01$ after 14 days), and while this is difficult to interpret, it is tempting to suggest that the L-form occurs in a more active metabolic condition when the IgG response is lowered. White blood cell counts on patients' blood showed a significant positive correlation with thymidine incorporation after 14 to 21 days' incubation ($p < 0.01$ after 21 days), suggesting that there is a white cell response when the L-form is metabolically active. The only other clinical laboratory results to show correlation were 5-nucleotidase ($p < 0.05$ after 21 days) and haemoglobin ($p < 0.05$ after 7 days).

In summary, our results are consistent with the view that an L-form exists in association with the erythrocytes of both normal and rheumatoid subjects, and that the degree of

Table 1. Comparison of ^{14}C-thymidine incorporation (d.p.m/0.2 ml culture/5×10^6 R.B.C. per mm^3) by cultures of erythrocytes from normal and rheumatoid subjects

Days' incubation	Mean (No. of observations)		Variance	
	Normal	Rheumatoid	Normal	Rheumatoid
0	2.4 (29)	2.0 (28)	12.1	6.9
7	45.3 (29)	44.9 (29)	122.6	460.4
14	42.4 (28)	54.0 (28)	41.8	422.1
21	47.9 (27)	52.6 (24)	91.9	376.5
28	53.1 (27)	54.5 (24)	143.4	507.1
35	62.0 (23)	54.3 (21)	356.7	809.3

Table 2. The effect of decreased numbers of leucocytes on ^{14}C-thymidine incorporation

Sample and method or preparation of suspension	R.B.C. (10^6 per mm^3)	W.B.C. /mm^3	^{14}C-thymidine incorporation (d.p.m./0.2 ml culture/5×10^6 R.B.C. per mm^3) after incubation for (days)					
			0	7	14	21	28	35
Normal subject								
Washed	4.06	8.0	5	41	55	54	48	43
Dextran-deposited	3.84	3.2	5	46	64	68	59	56
Dextran-deposited; frozen in glycerol	3.39	0	10	56	67	87	91	52
Rheumatoid patient								
Washed	4.08	3.4	5	42	49	45	48	42
Dextran-deposited	4.66	1.3	2	31	45	55	46	44
Dextran-deposited; frozen in glycerol	3.81	0	8	51	58	72	60	50

Table 3. The effect of deoxyribonuclease on ^{14}C-thymidine incorporation

Sample	Presence of deoxyribonuclease	^{14}C-thymidine incorporation (d.p.m./0.2 ml culture/5×10^6 R.B.C. per mm^3) after incubation for (days)					
		0	7	14	21	28	35
Lysed erythrocyte	–	12	27	33	69	27	35
Suspension prepared from normal subject	+	5	14	11	17	14	19

incorporation of thymidine by erythrocyte suspensions from rheumatoid patients shows a significantly greater variability than that from normal subjects. The control tests show that the incorporation is unlikely to be due to culture medium, contaminating platelets or leucocytes, or accidental microbial contamination, while the considerable reduction of incorporation by deoxyribonuclease very strongly suggests that incorporation is into DNA, almost certainly microbial, although the possibility that it is residual erythrocyte DNA cannot be absolutely excluded. We had anticipated that, in the event of incorporation of thymidine, this might correlate with the clinical activity of the rheumatoid patients, but this was not the case. However, the observation of a negative correlation with IgG and a positive correlation with the patients' white cell count are of considerable interest in view of the concept, often advanced, of rheumatoid arthritis being the result of inappropriate immunological responses to an infective agent.

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Dialyzable serum factors alter cellular immunity in pregnancy

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Summary. Lymphocyte responsiveness was found to be decreased in pregnant and increased in habitually aborting women. This is attributed to a dialyzable serum factor(s), that can be removed from the surface of lymphocytes by repeated washing.

Decreased cellular immunity in pregnancy may be due to either alteration in the proportion of lymphocyte subpopulations[1,2] or to certain blocking factors present in pregnancy sera[3,4] and absent from the serum of women with habitual abortions[5]. In the present work we attempted to obtain data about the approximate molecular size of the blocking factor, and investigated whether depressed reactivity depends entirely on blocking factors, and/or whether it has its basis in an intrinsic property of the lymphocytes.

Lymphocyte reactivity against embryonic antigen was tested in of 60 women (20–36 years old) including 20 women with a previous history of two or more idiopathic spontaneous abortions, without having successful pregnancies (AB group), 20 women pregnant for 13–30 weeks (PR group) and 20 nonpregnant women wearing intrauterine devices (IUD group). Venous blood was drawn from every subject into heparinized tubes. Lymphocytes were separated as described by Boyum[6], and divided into 2 parts; one part (LC-1) was washed once, the other part (LC-5) 5 times in 0.5 M phosphate buffered saline (PBS) and suspended in Hepes buffered minimal essential medium (MEM). Human embryonic fibroblast (HEF) cells, derived from 12 to 14 weeks old fetuses as described by Youngner[7] were used as target cells in the cytotoxicity assay and as stimulator cells in the experiments on production of migration inhibitory factor (MIF). MIF production by LC-1 and LC-5 of the 3 groups was compared. The method described by Rocklin et al.[8] was followed.

For testing MIF produced by lymphocytes, we used the guinea-pig macrophage migration inhibition assay according to Clausen[9]. It was found that LC-1 lymphocytes of the AB group produced significantly more MIF ($p < 0.02$) and those of PR group significantly less MIF ($p < 0.001$) than did LC-1 of nonpregnant women. In the case of LC-5, MIF production in all 3 groups was high, and no significant differences could be detected (fig. 1). These results indicate

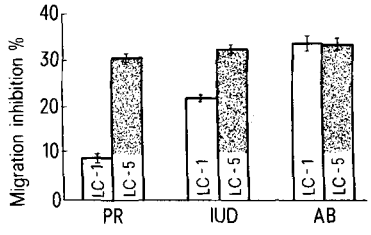

Fig. 1. Influence of exhaustive washing on MIF production by lymphocytes pregnant (PR), nonpregnant (IUD) and habitually aborting (AB) women. Lymphocytes were washed once (LC-1) or 5 times (LC-5) before stimulation. Each pair of columns indicates mean ± SE of 20 individuals.

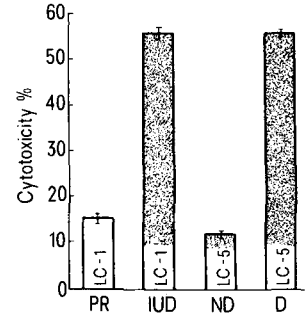

Fig. 2. Cytotoxicity of lymphocytes. The 1st 2 columns compare cytotoxic activity of lymphocytes from pregnant (PR) and nonpregnant (IUD) women. Cells were washed once (LC-1). Cytotoxic activity of lymphocytes from nonpregnant women (IUD), washed 5 times (LC-5) and than incubated in nondialyzed (ND) or dialyzed (D) pregnancy serum in shown in the last 2 columns. Means ± SE of 20 tests are indicated by each column.