J. WILLIAM HOLLINGSWORTH*
EUGENE R. SIEGEL**
WILLIAM A. CREASEY†

Departments of Internal Medicine and Pharmacology, Yale University School of Medicine, New Haven, Connecticut

GRANULOCYTE SURVIVAL IN SYNOVIAL EXUDATE OF PATIENTS WITH RHEUMATOID ARTHRITIS AND OTHER INFLAMMATORY JOINT DISEASES¶

The cellular constituents of synovial joint effusions of patients with rheumatoid arthritis present some curious features that may lead to an understanding of the nature of the disease. Effusions may be present constantly for years, with the total and differential cell count remaining essentially the same. If the fluid is removed by aspiration of the joint, the effusion rapidly recurs and tends to resume its original cellular make-up. The cells in the effusion are intriguing, because they are predominantly polymorphonuclear leukocytes in a chronic effusion in a disease in which the synovial histological picture presents typical features of chronic inflammation — lymphocytic and plasmocytic infiltration, chronic granulation tissue, and fibrosis.

This paradox of a chronic effusion containing mostly polymorphonuclear cells in a chronic inflammatory disease led us to postulate that these cells were, in effect, sequestered in the synovial cavity in a relatively adynamic state, with slow death of the exudative cells and a small daily replacement from blood granulocytes. To test this hypothesis, a marrow cell population of granulocytes was labeled by the induction of the enzyme dehydrofolate reductase by the folic acid antagonist, amethopterin (methotrexate). Following injection of methotrexate, the enzyme accumulates in dividing granulocyte precursors in the bone marrow, and their mature granulocyte progeny emerge in the blood as cells with increased enzyme content. As with other methods of bone marrow cell labeling, in normal subjects enzyme-rich cells are detectable in the blood in 3 or 4 days, peak at 7 to 9 days, and decline rapidly over the next week.[1]

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* Associate Professor of Medicine.
** Fellow in Medicine.
† Assistant Professor of Pharmacology.
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Patients with rheumatoid arthritis were found to have essentially normal granulocyte production, with peak levels in the blood at 7 to 9 days. If the granulocytes in the effusions were indeed sequestered and adynamic, one would expect little labeling of cells in the effusion during the "pulse" of enzyme-rich cells in blood. In the studies, however, blood and synovial effusion leukocytes were sampled simultaneously after methotrexate injection and similar levels of enzyme were found in cells from each source. This indicated that the cells in the effusions were far

TABLE 1. CLINICAL FEATURES OF PATIENTS IN THE STUDY

			Serum rheumatoid factor		*Synovial exudate*	
Patient	*Diagnosis*	*Sex*	*Latex*	*Sheep cell (units)*	*Total WBC/mm³*	*% Polys*
A.K.	R.A.	F	1:160	32	29,900	85
M.O'B.	R.A.	F	Pos.	128	26,000	43
W.A.	R.A.	M	1:160	—	7,700	81
I.R.	R.A.	F	Neg.	Neg.	50,800	86
L.T.	R.A.	M	1:320	—	10,400	51
S.O.	R.A.	M	Neg.	—	11,800	74
E.B.	R.A.	F	1:160	128	32,900	89
B.P.	R.A.	F	1:80	32	12,400	76
A.J.	R.A.	F	1:640	128	11,100	90
G.D.	Reiter's	M	Neg.	—	6,500	80
J.deL.	Reiter's	M	Neg.	—	56,600	91
W.W.B.	Ulc. colitis	M	Neg.	—	25,400	89
F.S.	Gout	M	Neg.	—	24,800	95
N.B.	Gout	M	—	—	11,400	97
M.K.	Gout	F	—	—	25,700	95

from adynamic, and were being regularly replaced by cells from the circulation.[1]

These observations demonstrated that the turnover of cells in rheumatoid synovial effusions was quite rapid, probably within a matter of a day or two. To more accurately define the rate of destruction of cells in the effusions, a method of labeling cells within the effusion was needed. This paper presents the results of tagging the cells in rheumatoid effusions with isotopically labeled diisopropylfluorophosphate (DFP). To determine whether the rapid turnover of granulocytes in synovial effusions of rheumatoid arthritis is unique to that disease, observations were made on a

smaller number of patients with other, more acute, synovial inflammatory diseases.

PATIENTS AND METHODS

Patients

Twelve experiments in nine patients with rheumatoid arthritis were performed. All patients had chronic arthritis with the effusions present for months or years. To compare with this group, three patients with acute gout, two with Reiter's syndrome, and one with the arthritis of ulcerative colitis were studied. Certain clinical features of the patients and of their synovial effusions are recorded in Table 1.

Standard clinical criteria (American Rheumatism Association) for the diagnosis of rheumatoid arthritis were employed, and all patients had advanced disease at the time of study. Serum rheumatoid factor was tested by the hospital laboratory, using both the sensitized sheep cell and latex tests. The patients with gout had hyperuricemia and characteristic needle-like urate crystals in the synovial exudate. Two patients with Reiter's syndrome had the classical triad of symptoms (arthritis and conjunctivitis following nonspecific urethritis), and recovered from the episode uneventfully in 3 to 6 months. The one patient with ulcerative colitis had associated spondylitis with obliteration of the sacroiliac joints radiographically and recurring synovitis of the knees.

Only patients who had knee joints with effusions in excess of 15 ml. in volume were studied. The clinical estimation of the volume of effusion was usually checked at the end of an experiment by complete aspiration of the joint.

Direct estimate of granulocyte lifespan by injection of labeled DFP into synovial effusions.

Initially, DFP^{32} was used in the studies, but later the tritium-labeled compound was used because of its long radioactive half-life. Approximately 30 μc of DFP^{32} (in 1 ml. volumes) or 30-100 μc of DFP-H^3 (in 0.03 to 0.1 ml. of propylene glycol) were injected into the synovial exudate. To insure mixing, fluid was flushed back and forth in the syringe several times, and after the isotope injection the patient was asked to extend and flex his knee repeatedly. One hour was usually allowed for binding of DFP to cellular and exudate proteins, and samples of 1 to 3 ml. volume were withdrawn from the knee at intervals thereafter. After initial trials with varied time intervals, a suitable sequence was found to be 1, 2, 4, 6 and 8 hours after injection. The leukocytes were washed four times in saline and freed from any contaminating erythrocytes incident to the puncture by lysing the latter with hypotonic saline. The last wash contained only a trace of radioactivity. For the experiments with DFP^{32}, the leukocytes were suspended in absolute ethanol, transferred to stainless steel planchettes, and allowed to evaporate to dryness at room temperature. Radioactivity was then measured with a Nuclear Chicago windowless gas-flow counter, and corrections applied for radioactive decay. After the counting procedure was complete, the residues in the planchettes were dissolved in sodium hydroxide solution (1 N) and the nitrogen content estimated by the Kjeldahl and biuret methods.[2] In studies with DFP-H^3, saline-washed labeled leukocytes were washed with cold perchloric acid (0.5M) to remove any small amounts of acid-soluble, presumably unbound, DFP, and the residue suspended in 0.2 N sodium hydroxide solution. Aliquots of the suspension

were counted in a Packard Tri-Carb Spectrometer with a toluene-ethanol scintillation fluid containing *p*-bis (s-5-phenyloxazolyl)-benzene and 2,5-diphenyloxazole.[8] The radioactivity derived from DFP-H^3 was expressed on the basis of the total number of leukocytes determined with a Coulter Model A particle counter. Radioactivity in the cell-free synovial exudate was also measured in some experiments.

RESULTS

Effect of the multiple aspirations on the cellular composition of synovial exudates

It was impossible to measure volume except by estimation, but none of the patients exhibited any clinically detectable change during the 8-hour period of most of the studies. Cell counts were obtained on almost all

TABLE 2. JOINT FLUID LEUKOCYTE COUNTS (CELLS/MM^3) IN SEQUENTIAL SAMPLES DURING THE COURSE OF DFP STUDIES

	Hours					
Patient	*1*	*2*	*3*	*4*	*5*	*6*
B.P.	15,800	18,100	19,300	18,500		
A.J.	12,200	9,700	10,800	9,600		
I.R.	32,800	31,500	34,900	41,000		
F.S.	19,200	16,200	15,200	12,600	12,500	16,100
J.deL.	56,600	52,400	73,600	54,200	51,800	54,600
N.B.	11,400	10,400	10,900	9,320	9,000	
W.W.B.	24,300	21,600	20,800	22,400	21,400	
M.K.	9,200	27,700	15,500	24,300		
G.D.	5,080	3,200	4,700	3,700	3,300	

of the samples in nine patients (Table 2). The first three patients in the Table are those with rheumatoid arthritis, and their counts were quite constant. One patient with Reiter's syndrome (G.D.) had a low initial cell count that seemed to fall with time. Two of the patients with acute gout (F.S. and N.B.) had surprisingly constant values, but the counts of one (M.K.) varied unpredictably and her initial low count suggests a laboratory error. Differential leukocyte counts were obtained in many of these patients, and showed no significant alterations during the course of the experiments.

Comparison of in vivo *and* in vitro *labeling in one patient.*

One patient was studied on three separate occasions. In one experiment (♯1 in patient A.K. in Table 3) 44 ml. of joint fluid was withdrawn into a siliconized syringe containing 30 μc of DFP^{32} and incubated *in vitro* for 30 minutes before the exudate was returned to the synovial cavity.

In this experiment, blood leukocyte samples were assayed for P^{32} at the same times as the joint fluid, and only a trace of cell-associated radioactivity was detected.* The disappearance rate from this *in vitro* incubation experiment in this patient was similar to values obtained in two later experiments in which the DFP was directly injected into the synovial cavity (Table 3).

TABLE 3. LOSS OF LABEL FROM CELLS IN SYNOVIAL EXUDATES

Patient	Date of study	Isotope	Per cent of leukocyte radioactivity after DFP injection (hours) 0.5	1	2	3	4	5	6	7	8	9	24
A.K.	3-20-63*	P^{32}	100			67				31		42	6
	5-20-63	P^{32}		100		80		45		46			
	2-11-64	H^3		100		61		23					3
M.O'B.	4-24-63	P^{32}	100	80		52				33			28
W.A.	5-17-63	P^{32}		100		60		56		40			18
I.R.	5-17-63	P^{32}		100		70		63		57			27
	3-24-64	H^3		100	79	67		57					
L.T.	6-18-63	P^{32}		100		62		57		49			
S.O.	6-18-63	P^{32}		100		56		20		22			
E.B.	6-18-63	P^{32}				100		55		16			
B.P.	3-18-64	H^3		100		94		63		19			0.5
A.J.	3-18-64	H^3		100		45		30		40			10
G.D.	7-28-65	H^3		100	47	85		53		35			
J.deL.	8-3-64	H^3		100	107	78		53		51			
W.W.B.	2-19-65	H^3		100	100	93		95		72			
F.S.	6-4-64	H^3		100	72	65		55		51			
N.B.	2-12-65	H^3		100	114	97		59		43			
M.K.	3-18-65	H^3		100	47	25				2			

* Isotope and exudate pre-incubated *in vitro* for 30 minutes before injection.

Loss of DFP-label from synovial exudate cells

In the 12 studies in patients with rheumatoid arthritis, 12 points were obtained 3 hours after the isotope injection; 10 points at 5 hours;

* From a calculation of the total circulating granulocyte pool, the largest amount of total blood leukocyte radioactivity (detected 30 minutes after the return of the synovial fluid-DFP mixture to the joint cavity) represented only 3% of the cell-associated DFP in the joint fluid. This slight blood cell activity probably represented DFP not bound to protein or cells in the synovial fluid, but free to diffuse into the circulation and attach to blood leukocytes. However, a small migration of cells back from the synovial cavity into the blood cannot be excluded. In any event, the amount of cell-associated label in blood leukocytes was so low that migration of tagged cells from blood to synovial exudate would not have added detectably to the joint fluid cell-associated radioactivity.

and 9 points at 7 hours. The mean values, with standard deviations, are shown in Figure 1. In the effusions of patients with rheumatoid arthritis, therefore, the granulocyte half-life seems about 4 hours. Although Figure 1 is plotted on an arithmetic scale on which the curvilinear disappearance suggests a random destructive process, the actual data are too variable to permit any statistical evaluation of the mechanism of destruction (random destruction or finite lifespan).

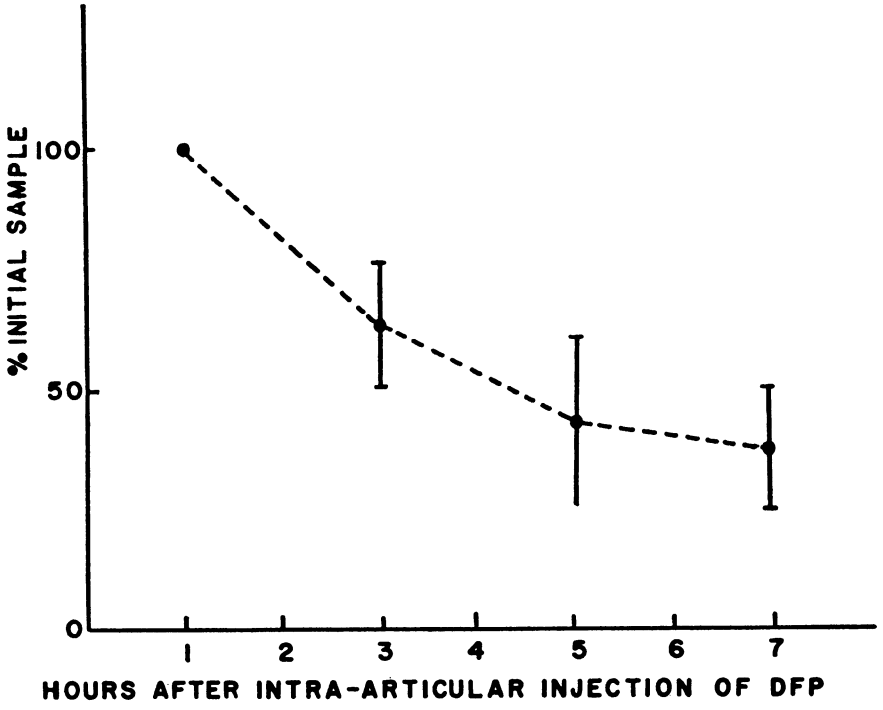

FIG. 1. Disappearance rate (mean ± S.D.) of labeled granulocytes from synovial effusions of patients with rheumatoid arthritis.

Although some of the patients had high titers of rheumatoid factor in their serum and marked morphological changes of vacuolization and degranulation in their synovial granulocytes, there was no gross difference in loss of cell-associated labeled DFP between those patients and the ones with negative rheumatoid factor tests and more normal granulocyte morphology. The two patients, A.K. and I.R., in whom more than one determination was available, are good examples. Both had chronic knee effusions present regularly for years and had similar rates of loss of cell label,

although the sero-negative patient (I.R.) never showed morphological changes on repeated examinations of exudative cells.

Similarly, of the three patients with overt gout and crystal phagocytosis only one (M.K.) seemed to have a somewhat rapid disappearance of cell-bound radioactivity in the effusion. The two patients with Reiter's disease (G.D. and J. deL.) also had loss of label from the cells in their synovial exudates similar to that of the patients with rheumatoid arthritis (Table 3).

DISCUSSION

To define further the rapidity of granulocyte destruction in rheumatoid effusions and to compare rheumatoid disease with other more acute synovial effusions, a method was needed for direct labeling of cells within the synovial cavity. Diisopropylfluorophosphate (DFP), labeled with either phosphorus^{32} or tritium, has been used to follow disappearance of granulocytes in the blood. Data of blood granulocyte half-life, obtained in this way, agree quite well with estimates obtained from granulokinetic data derived by other methods.[4] Since synovial exudate is free of erythrocytes and platelets, it seemed reasonable that DFP injected directly into the joint cavity would bind to granulocytes in the effusions. DFP does bind to mononuclear cells in blood,[5] but the synovial effusions are so predominantly polymorphonuclear that the errors introduced by labeling of other exudate cells are small.

There are several potential inadequacies in using this method to label cells in synovial effusions: 1) continuing attachment of DFP to cells over a period longer than the arbitrary one hour used in most of these studies; 2) re-utilization of the label; 3) loss of fragile cells during the repeated saline washes, a phenomenon that definitely occurs in rheumatoid fluid[6]; 4) inadequate mixing in the total effusion when the label is injected into the suprapatellar synovial extension only; and 5) labeling of mononuclear phagocytes. Undoubtedly much of the variability noted from point to point in the patients (Table 3) result from such problems as variable loss of cells during washing and inadequate *in vivo* mixing.

The inherent and uncontrollable aspects of the method—incomplete fixation of the label, re-utilization, tagging of mononuclear cells—all tend to give more prolonged values for cell-associated labels. Only if loose binding of the isotope occurred, a problem not encountered in the many studies of blood leukocyte disappearance time,[5,7] could the values obtained in these experiments be shortened spuriously. Therefore, it would

seem that these observations are a conservative estimate of true survival time of granulocytes in these effusions.

These observations in patients with diverse synovial inflammatory disease, then, probably indicate a rapid destruction of exudate granulocytes in synovial effusions, regardless of the inflammatory stimulus. The half-life of exudative granulocytes is only a few hours, with only a small proportion of the original cells remaining after 24 hours. In chronic effusions, therefore, there is a large daily destruction of cells and their replacement from the blood, a turnover at least as great as the total granulocytes in the effusions. In a quite average 30 ml. rheumatoid effusion containing 25,000 granulocytes/cu.mm., the daily breakdown in the synovial cavity might well exceed a billion cells.

SUMMARY

The lifespan of granulocytes in inflammatory synovial effusions has been estimated by labeling cells with diisopropylfluorophosphate (DFP). Twelve studies in nine patients with rheumatoid arthritis and six studies in patients with other inflammatory joint disease were performed. The half-life of the granulocytes was about four hours in the rheumatoid patients, and the disappearance rate was similar in the other types of inflammatory synovitis.

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