

# Cytotoxic Activity of Rheumatoid and Normal Lymphocytes against Allogeneic and Autologous Synovial Cells In Vitro

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**ABSTRACT** The possibility that lymphocytes from patients with rheumatoid arthritis (RA) might be sensitized to RA synovial cell antigens was investigated with a  $^{51}\text{Cr}$  release cytotoxicity assay. Peripheral blood lymphocytes from rheumatoid and normal donors were tested for cytotoxic activity against their own synovial cells and against allogeneic rheumatoid and nonrheumatoid synovial cells. In the allogeneic studies, the degree of cytotoxicity was significantly influenced by the age in culture (passage number) of the synovial target cells ( $P < 0.001$ ). When the passage number of the target cells was considered in the analysis, rheumatoid lymphocytes were found to have greater cytotoxic activity than normal lymphocytes against young cultures (low passage number) of both RA and non-RA synovial cells ( $P = 0.0042$ ). Differences in susceptibility to lysis between RA and non-RA synovial cells were detected only when cultures of high passage number were tested. In this situation, RA synovial cells were more susceptible to both RA and normal lymphocyte-induced lysis than were non-RA synovial cells ( $P = 0.0048$ ). No evidence of cytotoxicity was detected when lymphocytes from nine RA patients and two osteoarthritis patients were reacted against their own synovial cells. Although the data demonstrated an increased cytotoxic activity of peripheral blood lymphocytes from some RA patients against allogeneic synovial cells, the fact that this reactivity was seen against both non-RA

and RA synovial cells and was not demonstrated against autologous synovial cells argues against the presence of an immunospecific response of RA lymphocytes to RA synovial cell antigens.

## INTRODUCTION

Particular attention has recently been directed toward the role of cell-mediated immunity in the synovial inflammation characteristic of rheumatoid arthritis (RA).<sup>1</sup> This interest has been prompted by the observations of several investigators that suggest that the chronic joint inflammation of RA may result from local immune responses to antigens present in the synovial tissues (1, 2). Support for this concept includes the identification of perivascular accumulations of macrophages in close association with lymphocytes in various stages of transformation in rheumatoid synovial tissues (3) and the presence of immunoglobulin-producing plasma cells (4, 5), thymus-dependent lymphocytes (T cells) (6, 7), and lymphokines (8) in both the synovial membrane and the synovial fluid of rheumatoid patients. The most impressive correlation between cell-mediated immunity and rheumatoid joint inflammation has been the striking clinical improvement of patients with severe rheumatoid disease after depletion of circulating T cells by prolonged thoracic duct drainage (2, 9). The therapeutic benefits of immunosuppressive drugs, such as cyclophosphamide and azathioprine in the treatment of RA (10), also implicate

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<sup>1</sup>Abbreviations used in this paper: FBS, fetal bovine serum; MEM, minimum essential medium, M-H, Mantel-Haenszel; NF, nonrheumatoid synovial cells; NL, normal lymphocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; RA, rheumatoid arthritis; RAF, rheumatoid synovial cells; RAL, rheumatoid lymphocytes.

TABLE I  
*Source of Synovial Tissues and Donor Characteristics*

Source of specimen	Rheumatoid	Nonrheumatoid
Knee	4	4
Hip	1	17
Other (Hand, wrist, elbow)	10	2
Total number	15	23
Donor characteristics		
Age, yr; mean	54	59
range	38-72	29-80
Sex: female	11	13
male	4	10
Arthritic condition	Rheumatoid arthritis	Osteoarthritis—21 Other* — 2

\* One specimen was obtained from a patient with congenital hip dislocation and another from a patient with posttraumatic aseptic necrosis.

lymphocytes as significant elements in the pathogenesis of RA.

In vitro studies have suggested that lymphocytes from patients with RA express cell-mediated immunity to antigens present in rheumatoid but not in normal synovial tissues and fluid (11-13). Based on these preliminary reports, this investigation was undertaken to determine if a specific immune reactivity of rheumatoid lymphocytes toward rheumatoid synovial cells grown in tissue culture could be demonstrated in vitro. The cytotoxic activity of rheumatoid and normal peripheral blood lymphocytes against monolayer cultures of fibroblasts derived from rheumatoid and nonrheumatoid synovium has been compared with chromium-51 release assays.

## METHODS

**Source of synovial tissues.** Synovial tissue specimens were obtained from patients at the time of therapeutic surgical synovectomy or reconstructive joint surgery (Table I). Nonrheumatoid synovia were predominantly specimens obtained from the hip joints of patients with a clinical diagnosis of osteoarthritis. RA synovia specimens originated primarily from the knee, hand, and wrist joints of patients with definite or classical RA, based on the criteria of the American Rheumatism Association. The ages of the donors of rheumatoid and nonrheumatoid synovia were similar, while the proportion of women was higher in the rheumatoid group.

At the time of explanation, typical samples of dissected synovial tissue were fixed in formalin and examined histologically to confirm the presence of synovial membrane and the presence or absence of the inflammation, synovial cell hyperplasia, and lymphocytic infiltration characteristic of rheumatoid synovitis.

**Synovial cell cultures.** Monolayer cell cultures derived from the synovia of 38 patients (15 RA, 23 non-RA) were used in this study (Table I). Explant cultures of synovial lining tissue were established as previously described (14, 15). Initially, six explants of synovial tissue (approximately 1-2 mm in diameter) were seeded in each of several 25 cm<sup>2</sup> tissue culture flasks (Falcon, BioQuest, Oxnard, Calif.). Fibroblastic cells migrating from these explants were termed "pass 1" cells. When the monolayers reached confluency

(4-6 wk), the cells from two 25-cm<sup>2</sup> flasks were passaged to one 75-cm<sup>2</sup> plastic flask (1:1.5 split) and termed "pass 2" cells. Subsequent subculturing of individual cell lines was performed as the monolayers reached confluency (0.25% trypsin in phosphate-buffered saline) and were at split ratios of 1:2. All cell lines were maintained at 37°C, in a room air environment with Lebovitz medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories), glutamine (0.3 mg/ml), penicillin (100 U/ml), and gentamicin (40 µg/ml).

By continual subculturing, synovial cell lines could be maintained in culture for up to 2 yr (mean life-span, 10 mo), and thus provided a constant supply of synovial cells for study. The morphologic appearance and the growth characteristics of our synovial cell cultures were similar to those described by Bartfeld (16) and Castor and Fries (17).

Samples of synovial tissue specimens were monitored for bacterial contamination by inoculation into trypticase soy broth and for mycoplasma contamination as previously described (18). These tests were repeated on all cell lines in which a high cell-mediated cytotoxicity response was obtained in the <sup>51</sup>Cr-release assay and were routinely performed at 6-mo intervals on all synovial cell lines.

**Source and preparation of lymphocytes.** Lymphocytes from 40 Caucasian patients with RA (12 men and 28 women) were tested for cytotoxic activity against synovial cells in vitro. All patients were being followed in the Arthritis Clinic, University of Utah Medical Center, and the diagnosis of definite or classical RA was established according to American Rheumatism Association approved criteria. Tests for serum rheumatoid factor by latex fixation were negative in 15% of the patients. Drug therapy at the time of assay was restricted to aspirin and/or gold. The age of the patients varied from 21 to 80 yr (mean age 51) and the mean duration of disease was 11 yr (range 1-27 yr). 22% of the patients had undergone orthopedic surgery in the past, but none had been transfused. 25 healthy Caucasian laboratory personnel (9 women and 16 men) with ages ranging from 18 to 55 yr (mean age 32 yr) served as a source of normal lymphocytes for control studies.

Lymphocytes were prepared from heparinized (10 U/ml, preservative-free) peripheral blood samples by the Ficoll-Hypaque technique of Böyum (19) and washed three times in Hanks' balanced salt solution containing 5% heat-inactivated fetal bovine serum (FBS). Cell viability was determined by trypan blue dye exclusion (> 95%). Differential cell counting of Giemsa-stained smears demonstrated mononuclear cell populations consisting of 75-80% small to medium-sized lymphocytes and 20-25% large lymphocytes and monocytes. No attempt was made to remove adherent or phagocytic cells from the lymphocyte preparation.

**Cytotoxicity assays.** Cell-mediated cytotoxicity was assessed by <sup>51</sup>Cr release from labeled synovial cell monolayers (20). Monolayers of synovial cells were trypsinized (0.25% trypsin in phosphate-buffered saline [PBS] 30 min, 37°C) and washed twice with Eagle's minimum essential medium (MEM) (Hanks' salts), supplemented with 10% heat-inactivated FBS (MEM-FBS), glutamine (0.3 mg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). Washed synovial cells were resuspended in MEM-FBS and 2 × 10<sup>6</sup> cells in 1 ml were exposed to sterile Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> at 90 µCi/10<sup>6</sup> cells (initial sp act 191-429 mCi/mg, mean of 15 lots was 295 mCi/mg, New England Nuclear, Boston, Mass.) for 1 h at 37°C. All lots of <sup>51</sup>Cr were discarded after one half-life.

After  $^{51}\text{Cr}$  exposure, the cells were centrifuged at 200 *g* for 10 min and the supernates were carefully removed with suction and discarded. The labeled cells were then washed three times by resuspension in 10-ml volumes of MEM-FBS and centrifugation (200 *g*, 10 min). During the washing procedure, the MEM-FBS was kept at 37°C and all centrifugations were at room temperature. During preliminary studies, this procedure was shown to result in target cell suspensions in which 90–95% of the radioactivity was sedimentable at 200 *g* in 10 min and in which target cell viability was 90–94% by trypan blue dye exclusion. The washed, labeled cells were then resuspended in Dulbecco's medium supplemented with 15 mM Hepes buffer (pH 7.3, Flow Laboratories), glutamine (0.3 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% FBS (Dulbecco-FBS). Labeled cells,  $10^4$  in 0.2 ml medium, were added to the wells of Falcon Microtest II tissue cultures plates. The plates were covered with plastic film (Falcon, 3044) and incubated overnight at 37°C in a room air atmosphere. During this time, the cells attached to the bottom of the well as a confluent monolayer.

After 24 h, the medium was removed by rapidly inverting the plate and then blotting the plate surface on sterile gauze. Dehydration of the monolayers was prevented by quickly adding fresh medium (0.1 ml) to all but six wells. Lymphocytes ( $10^6$  cells in 0.1 ml media) were then added to the test wells and additional medium (0.1 ml) was added to the media control wells. The final volume of media and test wells was 0.2 ml. Trypsin (0.2 ml, 0.25% in PBS) was then added to the six reserved wells for later determination of the total radioactivity present in the monolayers. The plates were again covered with film and incubated for 18 h in a room air environment at 37°C.

The plates were then placed on a slight incline and the supernates were removed from one side of the well with a Pasteur pipet drawn to a fine point. Care was taken not to disturb the attached monolayers in the media and test wells. Each supernate was transferred to an individual counting vial and the amount of released  $^{51}\text{Cr}$  was measured in a crystal scintillation spectrophotometer (Biogamma, Beckman Instruments, Inc., Fullerton, Calif.). The trypsin wells were similarly harvested and, in addition, were rinsed three times with 0.2 ml distilled water. The rinses from each trypsin well were combined with the corresponding trypsin supernate for determination of the total radioactivity present per well. Microscopic observation of the trypsin wells confirmed lysis and removal of the target monolayer by this procedure. Assays were performed in replicates of six wells and 1 SD from the mean counts per minute was routinely less than  $\pm 10\%$  of the mean. The mean endogenous release of  $^{51}\text{Cr}$  from target cells cultured in the absence of lymphocytes (mean cpm of six media wells) during the 18-h incubation ranged from 15 to 22% of the mean total cpm per well. Total radioactivity per well, as determined from the mean of the six trypsin wells, ranged from 3,000 to 6,000 cpm. Percent lysis was calculated by the following equation: percent lysis = [(cpm of test wells) – (cpm of media wells)/(cpm trypsin wells) – (cpm media wells)]  $\times 100$ .

Synovial cells labeled with  $^{51}\text{Cr}$  formed stable monolayers under these assay conditions. The mean recovery of radioactivity (cpm) in the target monolayers (trypsin wells) after the initial 24-h preincubation was 85.6% of the cpm expected in the monolayer if 100% of the labeled cells had survived (mean of 15 determinations on different RA and non-RA synovial cell lines; range 60–95%). The expected cpm was calculated from the total cpm originally added to

the wells as cell-bound  $^{51}\text{Cr}$ , and an endogenous release of  $^{51}\text{Cr}$  into the media of 1%/h during this 24-h preincubation. Recovery of radioactivity in the target monolayer correlated well with the results of preliminary studies in which the relative recovery of unlabeled target cells was determined. In these studies confluent monolayers were trypsinized, washed three times in MEM-FBS, and resuspended in Dulbecco-FBS. Synovial cells,  $8 \times 10^4$ , in 2.5 ml of media were added to the wells of Linbro tissue culture trays (model FB-16-24-TC, Flow Laboratories) and incubated at 37°C. After 24 h, the wells were rinsed with PBS, the attached cells were removed with 0.25% trypsin in PBS, and total recovered cell counts per well were determined with a hemocytometer. The mean recovery of eight RA synovial cell lines was 83.5% (range 48–98%). The mean recovery of 11 non-RA synovial cell lines was 79% (range 38–97%). From these studies the average ratio of mononuclear cells to target cells in the  $^{51}\text{Cr}$  release assay was calculated to be approximately 120:1 and the maximum possible ratio was 250:1.

**Stimulation of lymphocytes with phytohemagglutinin (PHA).** Normal lymphocytes ( $10^6$ /ml in spinners MEM-FBS) were preincubated for 24 h at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air with and without PHA (2 µl PHA-P/ml, Difco Laboratories, Detroit, Mich.). The cells were then collected by centrifugation, resuspended in supplemented Dulbecco's medium, and tested for cytotoxic activity against  $^{51}\text{Cr}$ -labeled synovial cells as previously described. After 24 h, the medium was removed, and the percent release of  $^{51}\text{Cr}$  from the target cells affected by the PHA-prestimulated lymphocytes was compared with that of the unstimulated lymphocytes. In other assays, PHA was added directly to the lymphocyte-fibroblast cultures at this same relative concentration. Medium controls containing PHA demonstrated that this concentration of mitogen was not toxic for the target synovial cells.

**Statistical analysis.** The skewed distribution of values for percent lysis precluded the use of standard normal statistical procedures. For this reason, we used a distribution-free method, the Mantel-Haenszel (M-H) procedure (21) to test the independent effects of lymphocyte donor types (rheumatoid lymphocytes [RAL] vs. normal lymphocytes [NL]), synovial target cell types (rheumatoid synovial cells [RAF] vs. nonrheumatoid synovial cells [NF]), and age of target cell (measured by the number of passages in culture). For logistical reasons, we were unable to collect sufficient data to allow statistical analysis of effects of individual lymphocyte donors and individual target cell lines.

Two forms of scoring  $x$  and  $y$ 's, defined below, were used in the M-H procedure. One form was used to test the association between number of passages and cell lysis, controlled for lymphocyte donor type and target cell type (Table II). For each control factor combination (e.g., RAL and RAF), a one degree of freedom chi square was given by:  $M-H \chi^2 = [S - E(S)]^2 / V(S)$ ; where  $S = \sum xy$  with  $x$  = actual percent lysis and  $y$  = actual number of passages;  $E(S) = \sum x \sum y / T$ , the mean or expected value of  $S$ ,  $T$  = sample size, and  $V(S) = [\sum x^2 - (\sum x)^2 / T][\sum y^2 - (\sum y)^2 / T] / (T - 1)$ , the variance of  $S$ . The summations are over all observations available for the control factor combination.

The correlation coefficient,  $r$ , was obtained from M-H  $\chi^2$  by the equation:  $r = [M-H \chi^2 / (T - 1)]^{1/2}$ .

Summary chi squares, also with one degree of freedom, and  $P$  values were obtained by summing for the control factor categories to be combined thus: summary M-H  $\chi^2 = [2S - 2E(S)]^2 / 2V(S)$ .

TABLE II  
Correlation Coefficients between Number of Passages in Culture and Target Cell Lysis

Lymphocyte donor	Target cells		Summary values
	RAF	NF	
RAL	-0.22 (0.029)*	-0.25 (0.0035)	( $<0.001$ )
NL	0.09 (0.48)	-0.11 (0.33)	(0.94)
Summary values	(0.077)	(0.0028)	(0.0012)
<i>n</i>	180	220	

The Mantel-Haenszel (21) procedure was used to determine the effect of target cell age on susceptibility to lysis as a function of both lymphocyte type and target cell type. Test factors were: actual pass number of the target cells and percent lysis of the target cells by test lymphocytes, as judged by  $^{51}\text{Cr}$  release. The analysis was controlled for lymphocyte donor (RAL and NL) and target cell origin (RAF and NF).

\* Numbers in parentheses are *P* values of significance for association between low target cell pass number and elevated target cell lysis.

To test the independent effects of target cell type (Table III) and donor type (Table IV), the *x* scores were the same as above; however, for (a) target cell type  $y = 1$ , if RAF, and  $y = 0$ , if NF; and for (b) lymphocyte type  $y = 1$ , if RAL, and  $y = 0$ , if NL. In these last two analyses, (a) and (b), target cell passage was controlled with three ranges (2-5, 6-9, and 10 or more).

The lines presented in Fig. 3 were fitted by the method of least squares (22) to indicate trends. The scatter of points was so great, even after controlling for donor and target cell types, that these lines should not be construed to mean that we believe the relationship between percent lysis and target cell pass number to be linear.

## RESULTS

*Characteristics of synovial cells as targets for  $^{51}\text{Cr}$  release assays.* Studies were carried out to determine

the suitability of RA and non-RA synovial cells as targets for lymphocyte-mediated cytotoxicity assays. Since these cell lines are maintained as monolayer cultures and, therefore, undergo trypsinization before labeling with  $^{51}\text{Cr}$ , a standard 24-h incubation of  $^{51}\text{Cr}$ -labeled target cells was instituted to allow cell attachment to the surface of the wells and regeneration of any membrane antigens that might have been removed by the trypsinization procedure.

Rheumatoid and nonrheumatoid synovial cells were susceptible to lysis under assay conditions in which mitogen-stimulated lymphocytes were used as aggressor cells. In studies with normal lymphocytes pretreated with PHA, 85-90% of the bound  $^{51}\text{Cr}$  was released from the target cells during 24 h. PHA was also used to activate normal lymphocytes during 24 h of co-cultivation with  $^{51}\text{Cr}$ -labeled target cells. Under these conditions, the mean percent lysis at 24 h calculated from 23 determinations with 18 different synovial cell lines (10 non-RA and 8 RA) was 17% (range 7.7-30.2%) in the presence of PHA as compared to 4.6% (range 1.9-9.4%) in the absence of PHA.

*Comparison of the cytotoxic activity of rheumatoid and normal lymphocytes against allogeneic rheumatoid and nonrheumatoid synovial cells.* The cytotoxic activity of RAL and NL against allogeneic cell lines derived from both rheumatoid and nonrheumatoid synovium (synovial cells) were compared. In a typical assay, lymphocytes from one to three normal donors and one to four RA patients were simultaneously tested for cytotoxic activity against several RA and non-RA synovial cell lines. In the latter part of the study, both younger and older cell lines were included in each assay. The results of 400 assays are presented in Fig. 1. Although there was a tendency for high lysis values ( $>10\%$ ) to cluster in the rheumatoid lymphocyte groups, both the mean values of percent lysis (6.0-

TABLE III  
Mean Percent Lysis of RA and Non-RA Synovial Cells by RAL and NL as a Function of Target Cell Pass Number

Lymphocytes	Target cells	Target cell pass number		
		2-5	6-9	$\geq 10$
RAL	RAF	7.4 (0.3-38.7)* $n \dagger = 53$	4.23 (0.2-11.3) $n = 16$	6.06 (0.1-20.0) $n = 40$
RAL	NF	8.06 (1.2-47.2) $n = 72$	4.18 (0.5-12.6) $n = 34$	4.08 (0.1-9.7) $n = 27$
NL	RAF	5.38 (0.4-14.3) $n = 33$	5.41 (2.2-10.5) $n = 13$	6.01 (0.7-10.2) $n = 22$
NL	NF	5.11 (0.0-13.7) $n = 53$	4.0 (1.1-7.7) $n = 13$	4.29 (0.2-9.9) $n = 18$

\* Values in parenthesis represent range of percent lysis values obtained.

$\dagger n$  = number of assays with target cells of indicated pass numbers.

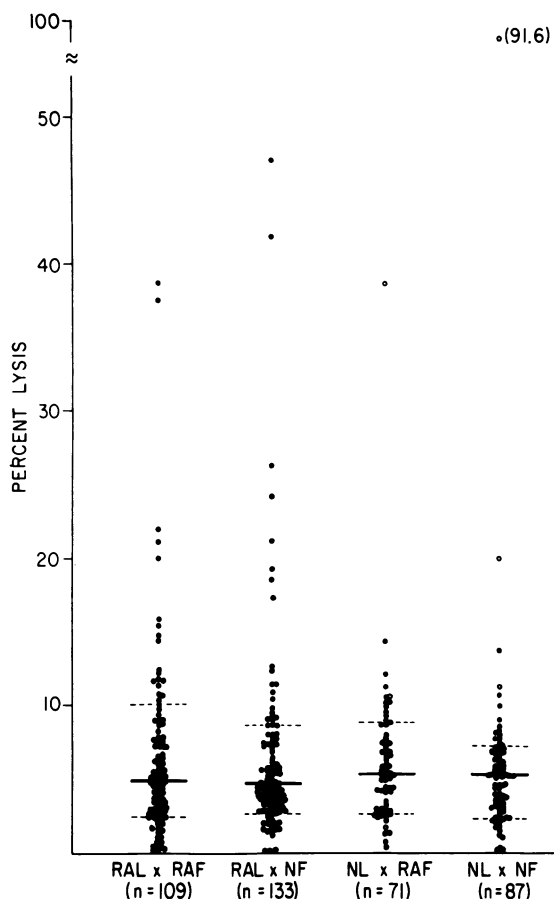


FIGURE 1 Results of  $^{51}\text{Cr}$ -release assays in which rheumatoid (RAL) and nonrheumatoid (NL) lymphocytes were tested for cytotoxicity against allogeneic rheumatoid (RAF) and nonrheumatoid (NF) synovial cells. Assays were as described in Methods with calculated aggressor-to-target-cell ratios of 120:1 and 18 h of incubation. The heavy solid lines denote the median values for percent lysis in each of the four groups. Dashed lines denote the 16 $\frac{1}{2}$  and 83 $\frac{1}{2}$  percentiles. The open circles represent the results of one normal individual discussed in the text.  $n$  = number of assays

6.4%) and the median values for location of the areas of central tendency (4.7–5.3%) were similar for all four combinations of lymphocytes and target cells.

The open circles (Fig. 1) represent the activity of lymphocytes from one normal individual whose lymphocytes were repeatedly cytotoxic for synovial and non-synovial cell lines in both FBS and human AB, Rho $^+$  serum. Serum from this male donor was negative for rheumatoid factor and anti-nuclear antibody. He did not have a history of arthritis but did experience recurrent bouts of bursitis. Since lymphocytes from this donor differed significantly from those of the other 24 normal donors, these values were excluded from the data in subsequent analysis.

TABLE IV  
Significance Values\* for the Relative Cytotoxic Activity against Synovial Cells of Rheumatoid vs. Normal Lymphocytes as a Function of Target Cell Origin and Age

Test factors	Target cells	Pass number of target cells			Summary values
		2-5	6-9	≥ 10	
% lysis					
RAL vs. NL	RAF	0.19	0.35	>0.9	0.35
RAL vs. NL	NF	0.010	0.89	0.83	0.017
Summary values		0.0042	0.62	>0.9	0.015

Control factors in the analysis were: age of synovial target cells in tissue culture as judged by pass number and synovial cell origin (RAF, rheumatoid synovial cells, NF, normal synovial cells). Test factors were: cytotoxic response of rheumatoid lymphocytes (RAL) vs. cytotoxic response of normal lymphocytes (NL) and actual percent lysis values, as determined by  $^{51}\text{Cr}$  release from the target cells in 18-h incubation of lymphocytes with target cells under assay conditions described in Methods.

\*  $P$  values of significance from chi square analysis as calculated by the Mantel-Haenszel procedure (21).

The cytotoxic activity of RAL and NL against allogeneic synovial cells as a function of target cell passage number *in vitro*. Upon scanning the individual assay

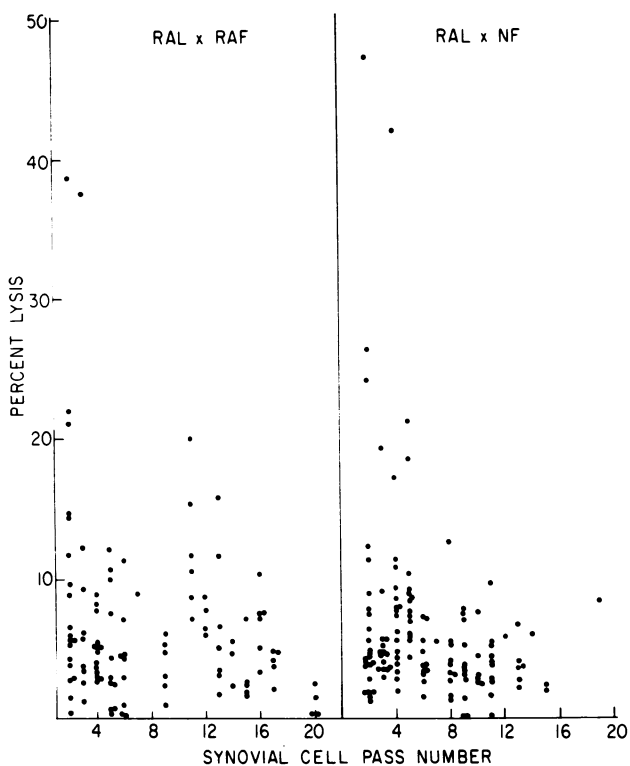


FIGURE 2 Percent lysis values obtained when rheumatoid lymphocytes (RAL) were tested for cytotoxic activity against rheumatoid (RAF) and nonrheumatoid (NF) synovial cells, as described in Methods, are plotted as a function of the respective target cell pass number at the time of assay.

TABLE V  
Significance Values\* for the Difference in Percent Lysis  
of Rheumatoid as Compared to Nonrheumatoid  
Synovial Cells as a Function of Target  
Cell Age and Lymphocyte Type

Test factors	Lym- phocyte	Pass number of target cells			Summary values
		2-5	6-9	≥10	
% lysis					
RAF vs. NF	RAL	0.64	0.9	0.038	0.23
RAF vs. NF	NL	0.74	0.20	0.044	0.068
Summary values		0.72	0.41	0.0048	0.65

Control factors were: age of target cells by pass number in tissue culture, and lymphocyte type (RAL, rheumatoid lymphocytes; NL, normal lymphocytes). Test factors were: target cell origin (RAF, rheumatoid synovial cells; NF, non-rheumatoid synovial cells) and actual percent lysis values as determined by  $^{51}\text{Cr}$  release.

\*  $P$  values from chi square analysis for significance of difference as calculated by the Mantel-Haenszel procedure (21).

results, it was noted that the higher values for percent lysis ( $> 10\%$ ) seen with RAL were observed primarily when synovial cells from very young cultures were used as target cells; i.e., lines subcultured only two to six times (Fig. 2). The data were subjected to statistical analysis to determine the significance of the effect of target cell age on the results of the cytotoxicity assays (Table II). Age of target cells did not significantly affect the results of cytotoxicity assays in which normal lymphocytes were tested ( $P = 0.94$ ). In contrast, rheumatoid lymphocytes demonstrated much greater cytotoxic activity against synovial cells from young cultures as compared to older cultures ( $P < 0.001$ ), and this association was significant for both rheumatoid ( $P = 0.029$ ) and nonrheumatoid ( $P = 0.0035$ ) synovial cells.

Since the number of times that the synovial cells had been subcultured appreciably influenced the frequency and extent of target cell lysis affected by rheumatoid lymphocytes, the assay results were grouped according to target cell age, as shown in Table III. Two analyses were then made that controlled for the age of the target cells. In the first analysis (Table IV), the cytotoxic activity of RA lymphocytes for synovial cells was compared with that of non-RA lymphocytes. In the second analysis (Table V), the susceptibility to lysis of RA vs. non-RA synovial cells was compared.

Significance values from a comparison of the relative cytotoxic activity of rheumatoid versus normal lymphocytes when tested against young, medium, and older synovial cell cultures are presented in Table IV. In the summary analysis, the overall cytotoxic activity of rheumatoid lymphocytes against synovial cells was significantly greater than that of normal lymphocytes ( $P = 0.015$ ). When the significance values calculated for the three target cell age groups were compared, however,

this apparently greater cytotoxic activity of rheumatoid lymphocytes was found to be highly significant only when young target cells (pass 2-5) were considered ( $P = 0.0042$ ) and was not significant when target cells from older cultures were tested ( $P = 0.62$ ;  $P > 0.9$ ).

Since differences in the cytotoxic activity of rheumatoid and normal lymphocytes were most prominent when young synovial target cells were used, it was expected that any disease-specific immune response of rheumatoid lymphocytes to rheumatoid synovial cells would be seen with young target cells. Therefore, a similar analysis to compare the lysis of RA vs. non-RA synovial cells of equivalent age in culture by each of the two lymphocyte types was made (Table V). RA lymphocytes did not demonstrate increased cytotoxic activity against young RA synovial cells as compared to young non-RA synovial cells ( $P = 0.64$ ). Thus, although rheumatoid lymphocytes were more cytotoxic when tested against young synovial cells than normal lymphocytes, no increased interaction between RA lymphocytes and RA synovial cells was demonstrated.

The data presented in Table V, however, show that differences in susceptibility to lysis between RA and non-RA synovial cells do arise after maintenance in tissue culture for several generations. Both rheumatoid and normal lymphocytes showed significantly greater cytotoxic activity against older RA cell cultures than against older non-RA cell cultures ( $P = 0.0048$ ). This finding suggests a difference between RA and non-RA synovial cells in vitro, evidenced primarily in older cell cultures and detected by both rheumatoid and normal lymphocytes.

Although in the group analysis presented in Table IV, RA lymphocytes were shown to have a greater cytotoxic activity against synovial cells than normal lymphocytes, it was readily evident that lymphocytes from the majority of patients with RA were nonreactive in the cytotoxicity assays. To identify those patients whose lymphocytes were repeatedly cytotoxic against synovial cells, lymphocyte donors were arbitrarily scored as high or low reactors in this assay system on the basis of demonstration of 11% or more lysis toward a minimum of two different synovial cell lines. By these criteria, 8 of the RA donors (20%), but only 1 of the 25 normal donors (4%), were considered high reactors. The effect of age in culture on the percent lysis values obtained when lymphocytes from the reactive rheumatoid group (R-RAL) and the nonreactive group (NR-RAL) were tested against RA and non-RA synovial cells is presented in Fig. 3. These data substantiate and graphically demonstrate the conclusions drawn from the statistical analyses of the entire RA patient group. Lymphocytes from these eight patients were found to be reactive to young cultures of both RA and non-RA

synovial cells. When results of simultaneous assays with lymphocytes from these same patients and older synovial target cells were compared, greater lysis of RA target cells than of non-RA target cells was observed.

We were unable to differentiate these eight patients from the RA patient group as a whole on the basis of age (mean 48 yr; range 21–64 yr), sex (three men, five women), duration of symptoms (mean 8 yr; range 1–16 yr), number of pregnancies among the female patients (none to three), drug regimen (aspirin, three patients; gold, five patients). Three of these eight patients had undergone major surgery in the past, and none had received blood transfusions. Two patients were sero-negative, and six were sero-positive for rheumatoid factor by latex fixation tests.

**Test of rheumatoid lymphocytes for cytotoxic activity against autologous synovial cells.** The cytotoxic activity of rheumatoid and nonrheumatoid lymphocytes against autologous synovial cells was investigated in 11 patients (Fig. 4). In each case, the autologous synovial target cells under consideration were at pass 2–6. Control lines included young allogeneic RA and non-RA synovial cells. Lymphocytes from none of the nine RA donors demonstrated significant cytotoxic activity against either autologous synovial cells or against older normal

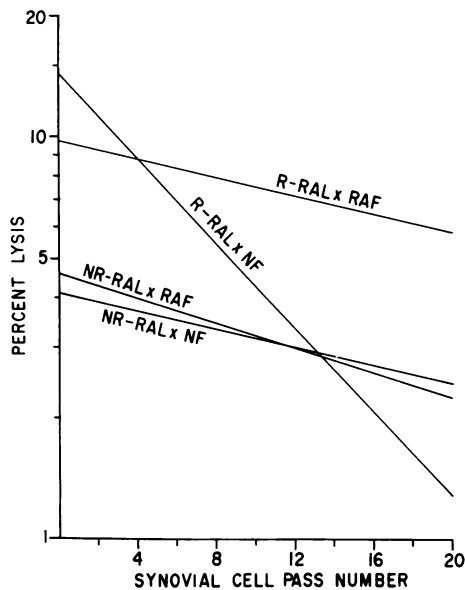


FIGURE 3 A linear relationship was fitted by the method of least squares (22) between the age of the target cells as judged by the number of passages in culture at the time of testing and the percent lysis of rheumatoid (RAF) and normal (NF) synovial cells affected by lymphocytes from eight rheumatoid patients considered high reactors in this assay system (R-RAL). Regression lines similarly calculated from the lytic responses of the remaining rheumatoid donors (NR-RAL) are shown for comparison. The plot is semilogarithmic and has been extrapolated to zero.

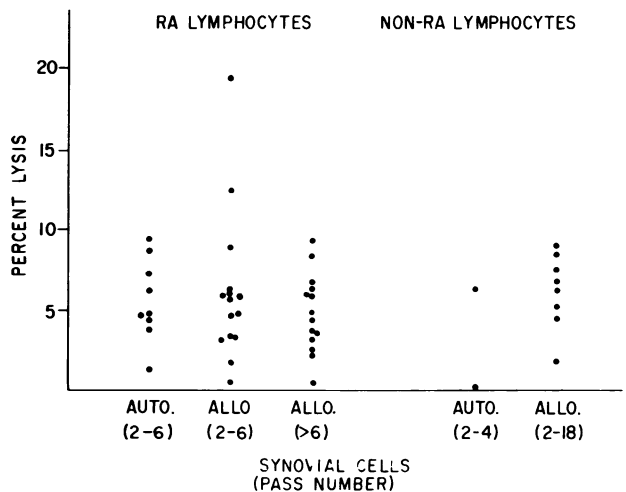


FIGURE 4 Cytotoxic activity of lymphocytes from nine patients with rheumatoid arthritis (RA) and two patients with osteoarthritis (OA) against autologous (AUTO) and allogeneic (ALLO) synovial cells under assay conditions as described in Methods. Numbers in parentheses represent age of target cells, as determined by number of passages in tissue culture, at the time of assay.

lines, although lymphocytes from two of these same donors did show increased cytotoxic activity against young allogeneic synovial cells. In control studies in which lymphocytes from two patients with osteoarthritis were tested against autologous and allogeneic synovial cell lines, no significant lysis of target cells was observed (Fig. 4).

## DISCUSSION

As pointed out in the introduction, rheumatoid synovitis may be due to local humoral and cell-mediated immune responses to antigens present in the inflamed synovial tissues (1, 2). Our study was initiated in an attempt to identify, in fibroblast cultures derived from the synovia of rheumatoid patients, an antigen (either foreign or self) that might have an etiologic or disease-perpetuating relationship to RA. Although we could not demonstrate a specific immune response of rheumatoid lymphocytes toward rheumatoid synovial cells, the results are of interest in that they demonstrate differences both between rheumatoid and normal lymphocytes and between rheumatoid and nonrheumatoid synovial cells.

Previous attempts to demonstrate immunity in RA patients to antigens unique to rheumatoid synovial tissue by in vitro techniques have been for the most part inconclusive. For example, other investigators have utilized fresh synovial tissue and synovial fluid as an antigen source in demonstrating disease-related immune reactivity in RA patients by lymphocyte blast transformation or leukocyte migration inhibition assays (11–13). However, the possibility that the immune responses ob-

served in vitro were due to immune complexes or pre-existing lymphokines arising from in vivo immune activity in the synovium was not excluded.

The cytotoxic activity of rheumatoid lymphocytes when tested against human cell lines has been investigated by a number of groups with conflicting results. Initial reports that peripheral blood lymphocytes from rheumatoid patients were cytotoxic for human synovial cells (23) and human embryonic fibroblasts (24) were unconfirmed by later investigations (25–28). In these initial studies, long-term incubations (48–72 h) of lymphocytes with allogeneic target cells were employed, and the possibility that the observed target cell lysis resulted from in vitro sensitization of lymphocytes toward target cell alloantigens (29) was not excluded. In contrast, Hedsberg (27) used morphological criteria to assess cytotoxicity after 24 h of incubation of rheumatoid lymphocytes with human fetal skin, human fetal kidney, and rheumatoid synovial membrane-derived fibroblasts. He observed that mononuclear cells from joint fluid, but not from peripheral blood, were cytotoxic for these target cells. Similar results were obtained by MacLennan and Loewi (25, 28) who, using a  $^{51}\text{Cr}$ -release assay and Chang liver target cells, detected cytotoxic activity by RA joint fluid mononuclear cells but not by RA peripheral lymphocytes. Fraser and Clarris (26) reported that both RA and normal peripheral leucocytes were cytotoxic for synovial cells but attributed these results to the presence of polymorphonuclear leucocytes.

Recent assessments of the cytotoxic activity of rheumatoid lymphocytes against synovial cells (30, 31) have also given conflicting results. Person et al. (30), using a microcytotoxicity assay, observed that the cytotoxic activity of rheumatoid lymphocytes against allogeneic synovial cells was greater than that of lymphocytes from healthy individuals and also that the cytotoxic response of rheumatoid lymphocytes was greater when rheumatoid synovial cells were used as target cells than when nonrheumatoid synovial cells were employed. In contrast, Ghose et al (31), using morphological criteria to assess cytotoxicity, studied the interaction of peripheral blood lymphocytes from rheumatoid and osteoarthritis patients with autologous synovial cell cultures. In this situation, immune responses to target cell alloantigens were obviated and no significant cytotoxic activity was observed in 24 h when rheumatoid lymphocytes were tested against autologous rheumatoid synovial cells.

In our study, lymphocytes from the majority of rheumatoid patients demonstrated no greater cytotoxic activity against synovial cells than did lymphocytes from healthy individuals. On closer examination of the data, it was found that the culture age of the synovial target

cells, as judged by the number of passages in culture before assay, directly influenced the individual assay results when rheumatoid lymphocytes were tested ( $P < 0.001$ ) but not when normal lymphocytes were tested ( $P = 0.94$ ). When the data were analyzed controlling for the culture age of the target cells, rheumatoid lymphocytes were found to have significantly greater cytotoxic activity against young cultures of allogeneic synovial cells than did normal lymphocytes, and our data are thus in accord with this aspect of Person's report. However, since RA lymphocytes were cytotoxic for young cultures of both RA and non-RA synovial cells, our data did not confirm the presence of a specific immune reactivity of rheumatoid lymphocytes toward cells derived from rheumatoid synovia. The data from our allogeneic studies thus suggest that RA lymphocytes may be presensitized to normal synovial antigens and that these antigens are most evident on young synovial cell cultures. This type of autoimmunity to normal self antigens could conceivably develop secondary to joint destruction, as a result of prior or chronic joint infection, or be due to abnormalities of the immune system like those in systemic lupus erythematosus (32).

Although the series is smaller, our autologous studies do not totally support the conclusion drawn from the allogeneic studies and are, in fact, similar to those of Ghose et al. (31). None of the lymphocyte preparations from nine patients with RA demonstrated significant cytotoxic activity against autologous synovial cells even though lymphocytes from two of these patients did show increased cytotoxic activity against young allogeneic synovial cells. These data, if confirmed in a larger series of autologous studies, would indicate that the allogeneic cytotoxicity assays may not have measured presensitization of RA lymphocytes to synovial tissue specific antigens, but were detecting a response to alloantigens present on the synovial cell membranes. One explanation for these results would be the presensitization of lymphocytes from some RA patients to human lymphocyte antigens as a result of pregnancy or blood transfusions; however, we were unable to correlate increased cytotoxic activity with either of these factors in the eight patients whose lymphocytes were most reactive in the allogeneic assays.

A second interpretation of this data would be that the observed target cell lysis resulted from primary in vitro activation of RA lymphocytes in response to target cell alloantigens. Since normal lymphocytes did not show this response under similar assay conditions, it is possible that some RA patients may possess a more rapidly reacting or a generally more aggressive population of peripheral blood lymphocytes than normal individuals. Hyperreactivity of rheumatoid lymphocytes in response to antigenic stimulation has not previously



been reported, and this interpretation should be viewed with caution until further studies designed to investigate this question are available.

The phenomenon referred to here as an "age effect" is a functional description, defined first by the observed increased cytotoxic response of RA lymphocytes to young cultures of allogeneic synovial cells and second by the greater susceptibility to lysis of older RA as compared to older non-RA synovial cell cultures. This effect could result from differences in antigenic expression or in physiologic integrity between younger and older cell cultures. Rapid loss of tissue specific antigens (33) and decreased expression of transplantation antigens (34) have been documented for other nontransformed cell lines in tissue culture. The decreased lysis of older synovial cells by RA lymphocytes may reflect a similar loss of antigenic stimulus in the target cell monolayer. Alternatively, the age effect might be due to metabolic or physiologic changes in synovial cell lines approaching senescence that result in an apparent resistance to lysis. The metabolic abnormalities of rheumatoid synovial cells, observed by other workers (35), may effect a slower rate of change in the antigenic or physiologic characteristics of RA as compared to non-RA synovial cells in culture and thus result in the observed target cell differences we have noted between older RA and older non-RA synovial cells. A third possibility is that the decreased lysis of target cells from older synovium-derived cell cultures might be due to an overgrowth of these cultures with a non-synovial cell type. The extension of this consideration is that due to the *in vivo* proliferation of synoviocytes in rheumatoid synovia, RA-synovial cell cultures might contain a greater percentage of true synovial cells initially and as a result exhibit a slower rate of overgrowth *in vitro*. Our data at present do not differentiate among these possibilities.

In summary, our study demonstrates that lymphocytes from some rheumatoid patients show greater cytotoxic activity against young allogeneic synovial cells than do normal lymphocytes, as measured by  $^{51}\text{Cr}$  release from the target cells in 18 h. However, we found no evidence for specific lysis of RA synovial cells by RA peripheral lymphocytes. The age in culture of the synovial target cells was shown to influence greatly the extent of observed lysis and thus should be considered in analysis of data arising from studies of this type. Data from the allogeneic studies could be interpreted on the basis of immunity to normal synovial cell antigens. However, the autologous studies did not support this conclusion and indicated that the results may have represented *in vitro* activation of generally more aggressive RA lymphocytes. Further autologous studies, in conjunction with an investigation of the effects of age in culture on

the susceptibility of synovial cells to cell-mediated lysis, will be necessary to differentiate between these two possibilities.

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