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Research Article

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The Cytotoxicity of Leukocytes and Lymphocytes from Patients with Rheumatoid Arthritis for Synovial Cells

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ABSTRACT Unseparated peripheral blood leukocytes obtained from patients with rheumatoid arthritis (RA) were cytotoxic for synovial cells. The cytotoxic reactions produced by RA leukocytes were more frequent and of greater magnitude than cytotoxicity induced by leukocytes from normal persons and patients with other diseases, primarily connective tissue diseases. Furthermore, the cytotoxic activity of RA leukocytes was greater for RA synovial cells than for nonrheumatoid synovial cells, in contrast to the cytotoxicity of other leukocytes, which did not discriminate between synovial cells according to their origin. Tests with purified lymphocytes showed that the cytotoxicity of unseparated leukocytes directed against RA synovial cells was due to lymphocyte cytotoxicity. These data are consistent with the possibility that sensitized lymphocytes from patients with RA recognize a distinctive antigen present on rheumatoid synovial cells.

INTRODUCTION

The etiology of rheumatoid arthritis (RA)[1] remains obscure. During the last several years a number of cell strains derived from both RA and nonrheumatoid (NR) synovial membranes have been established from explants and studied for viruses and mycoplasmas. Neither mycoplasmas nor viruses were found by direct tests, interference tests, or by radio-labeling the nucleic acids (1–4). To further examine RA synovial cells for an infectious agent, synovial cells have been used as target cells in cytotoxic tests, by the reasoning that if a virus, mycoplasma, or induced antigen were present on synovial cells, RA lymphocytes might be cytotoxic to synovial target cells. These methods should allow for the detection of any synovial cell differences, if present, irrespective of the origin of the antigen(s); i.e., be it a neo-antigen, auto-antigen, microbial, transplantation, tissue specific, fetal, or other antigen.

With both unseparated peripheral blood leukocytes and isolated peripheral blood lymphocytes, comparisons were made between aggressor cells from patients with RA, other diseases, primarily connective tissue diseases (CTD), and normal persons, and between RA and NR synovial cells as target cells. This report presents data that demonstrate that lymphocytes from patients with RA were significantly more toxic for RA-derived synovial cells than for NR synovial cells.

METHODS

Target cell cultures. Cell cultures were established as previously described, with Eagle's minimum essential medium containing 10% fetal calf serum, 0.75 g/liter sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 U/ml mycostatin (1, 4). The synovial cells were derived from 18 patients with RA, two patients with degenerative joint disease, two patients with aseptic necrosis of the femoral head (one with gouty arthritis and the other with hemoglobin sickle cell disease), two patients with noninflammatory knee effusions (one thought to be secondary to trauma, the other thought to have pseudogout clinically), and one patient with an osteocartilaginous loose body in the knee (secondary to trauma). Human embryonic lung (HEL) fibroblasts were studied to allow comparisons with a nonsynovial fibroblast target cell. In the tests with puri-

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During the earlier phase of these investigations, Dr. Person was a recipient of an Arthritis Foundation Postdoctoral Fellowship and is presently a Senior Investigator of the Arthritis Foundation.

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[1] *Abbreviations used in this paper:* CTD, connective tissue disease; HEL, human embryonic lung; HLA, histocompatibility antigens; NR, nonrheumatoid; RA, rheumatoid arthritis.

fied lymphocytes, a fibroblast cell strain designated AND, derived from the involved skin of a patient with systemic lupus erythematosus, was also used as a target cell. In spite of the variety of cell strains studied, they all have some morphologic characteristics of fibroblasts. Morphologically, one cannot distinguish the rheumatoid from the NR synovial cells. However, the HEL and AND cells are easily distinguishable from the synovial cells, as they have the morphology of classical fibroblasts. They are spindle-shaped, oriented in parallel, and produce a whorled pattern when confluent. In contrast, synovial cells are more heterogenous, exhibit pleomorphism and a more disoriented monolayer at confluence. The growth characteristics of synovial cells from different sources are indistinguishable in our experience. The HEL and AND cells have more rapid doubling times.

Synovial tissues obtained at surgery or biopsy were minced and explanted in plastic flasks (1, 4). After the outgrowth of cells from explant cultures was nearly confluent, the cells were trypsinized and split. This first split was designated as the first passage. All succeeding splits were made at confluency at 1:2 split ratios and numbered consecutively. None of the target cell strains studied was transformed. All have a finite life-span in culture. The designation, origin, and passage level at which the target cells were tested in the cytotoxic assay are listed in Table I. The plating efficiency of the target cells determined at the time of the cytotoxic assay was 15.8±1.1% in 60 tests with RA target cells and 18.2±2.0% in 24 tests with NR target cells.

Source and preparation of leukocytes. Peripheral blood leukocytes were obtained from 39 patients with RA; 12 normal persons; and 30 patients with other diseases, including 10 with systemic lupus erythematosus, 7 with gout, 3 with ankylosing spondylitis, 2 with progressive systemic sclerosis, and 1 each with degenerative joint disease, lumbar disc syndrome, osteopenia, chronic polyarthritis of unknown etiology, hepatitis B-induced periarteritis nodosa, Reiter's syndrome, Goodpasture's syndrome, and Behçet's syndrome. 10–15 ml of blood were collected with preservative-free heparin, 8 U/ml of blood. Red cells were allowed to settle by gravity for 1–3 h at 36°C, and the leukocytes were collected from the plasma and washed three times with Hanks' balanced salt solution. The concentration of white blood cells in the final suspension was adjusted to 2×10^6 viable cells/ml after a sample was counted and tested for viability with trypan blue.

Source and preparation of purified lymphocytes. Lymphocytes were obtained from patients with RA and normal laboratory personnel. 6–8 ml of heparinized whole blood (8 U heparin/ml blood) were layered on 5 ml of Ficoll-Hypaque, prepared by mixing 24 parts of an aqueous 9% (wt/vol) solution of Ficoll (Sigma Chemical Co., St. Louis, Mo.) with 10 parts of a 33.9% (wt/vol) aqueous solution of Hypaque sodium (brand of sodium diatrizoate, Winthrop Laboratories, New York). The specimens were centrifuged at 400 *g* in a swinging bucket rotor for 20 min at ambient temperature (5). The white band of mononuclear cells was collected and the cells were washed three times with Hanks' balanced salt solution with centrifugations at 50–70 *g* for 10 min each. After the third wash, the cell pellet was suspended in 1 ml of Eagle's minimum essential medium, supplemented with sodium bicarbonate, antibiotics, and 10% fetal bovine serum. All glassware used to this point was siliconized (Siliclad, Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). The cell suspensions were then allowed to attach to the walls of 1-oz soft glass prescription bottles at 36°C for 30–60 min. Nonadherent cells

TABLE I

Designation, Origin, and Passage Level of the Target Cells

Designation	Origin	Passages tested
BLU	RA synovial cell	4, 5, 7–10, 13, 16, 27
BRI	RA synovial cell	6, 8, 13
CAN	RA synovial cell	6, 15, 17, 19, 26
GRI	RA synovial cell	3, 5, 11, 13
HOP	RA synovial cell	6–11, 13, 16
KOH	RA synovial cell	6, 7, 12, 14
LYO	RA synovial cell	8, 9, 11, 14, 19, 25
MCM	RA synovial cell	5, 7, 9, 10, 12, 15
PON	RA synovial cell	6, 8, 14
REI	RA synovial cell	7, 10, 13, 16, 18
ROB	RA synovial cell	22
SER	RA synovial cell	4, 9, 10, 12, 14
TRI	RA synovial cell	8
VAL	RA synovial cell	6, 9, 10, 12, 15, 16, 18–20
WAL	RA synovial cell	6, 10
WER	RA synovial cell	7–10
WIL	RA synovial cell	5
WYN	RA synovial cell	5, 8, 21
ABR	NR synovial cell	17–19, 22
BAK	NR synovial cell	15, 17–21, 23, 26–28
BLA	NR synovial cell	14
LAN	NR synovial cell	4–7, 9, 10, 18
LOC	NR synovial cell	21, 23, 25, 29, 31
NEW	NR synovial cell	13
VIN	NR synovial cell	7–10, 12, 14, 16, 18–20
AND	SLE, skin fibroblast	4, 6
HEL	Human embryonic	18, 19, 21–24, 27
	Lung fibroblast	28, 30, 36

SLE, systemic lupus erythematosus.

were collected and counted in a hemocytometer. The final cell suspensions were adjusted to contain 2×10^5 viable lymphocytes/ml in Eagle's complete medium. Smears made from a centrifuged sample were stained with Wright's stain. In several tests, 0.1 ml of the suspensions was mixed with 0.1 ml of a 1:100 dilution of latex particles (Bacto latex 0.81, Difco Laboratories, Detroit, Mich.) and incubated overnight. Phagocytic cells were enumerated in smears prepared the following day. The morphology of a minimum of 200 cells was evaluated by light microscopy, and polymorphonuclear leukocytes, phagocytic, and nonphagocytic mononuclear cells were counted. Polymorphonuclear leukocytic contamination was approximately 2%. Morphologically, the isolated cells were primarily small lymphocytes, phagocytic cells constituting less than 2.5% of the total.

Microcytotoxicity assay: leukocytes. The target synovial and HEL cells were seeded in tissue culture grade, flat-bottomed, 96-well microtiter plates (Microtest II, Falcon Plastics, Division of BioQuest, Oxnard, Calif.) at a density of 200 viable cells/well in 0.01 ml of medium. The plates were covered and incubated overnight in humidified 8% CO_2 in air at 36°C. Usually 1 day's test included six plates, of which three were seeded with three different RA-derived synovial cells, two with different NR-derived synovial cells, and one with HEL cells. After overnight incubation, 10 µl

of the stock leukocyte suspension containing 2×10^6 cells/ml was added to six wells of each microtiter plate containing the different target cells, giving a leukocyte to target cell ratio of 100:1. The stock leukocyte suspensions were diluted 10-fold and were added to an additional six wells on each plate, giving a leukocyte-to-target cell ratio of 10:1. 10 μl of medium was added to 12 wells in each plate to serve as a control, and 10 μl of a 1:50 dilution of normal guinea pig serum in medium was added to each of the wells. Although it was subsequently established that fresh guinea pig serum was not required, to keep the conditions constant, guinea pig serum was included in all tests with unseparated leukocytes. The plates were covered and reincubated for 1 h, after which 0.2 ml medium was added to each well and the plates were incubated for an additional 24 h. The following day the medium was aspirated from each well, the wells were washed three times with Tris-buffered saline, and 10% formalin-saline, pH 9, containing magnesium carbonate, was added to each. The cells were stained with safranin O:safranin bluish (2.5 g safrinin O and 2.5 g safranin bluish in 100 ml 95% ethanol, filtered and diluted 1:1 in formalin-saline). The number of single-target cells remaining in each well was counted with the aid of an inverted microscope ($\times 65$ or $\times 100$). Two or more target cells clumped together were counted as one, on the assumption that the target cells had been clumped at the time of planting and had not had sufficient time for even one synovial cell doubling.[2]

Microcytotoxicity assay: lymphocytes. Synovial target cells were seeded and incubated as in the leukocyte tests. The following day the Ficoll-Hypaque-separated lymphocytes were suspended in medium to contain 2×10^5 lymphocytes/ml and 0.1 ml was added to six wells containing each target cell strain, giving aggressor: target cell ratio of 100:1. Each test usually included lymphocytes from five patients with RA and two control subjects. Eagle's medium was added to six wells containing target cells only, which served as target cell controls. No guinea pig serum was used in any test with lymphocytes. All of the plates were covered and reincubated overnight. The microtiter plates were processed as described for the leukocyte microcytotoxicity assay except that safranin O (2.5 g safranin O per 100 ml 95% ethyl alcohol, diluted 1:10 in distilled water and mixed with an equal volume of formalin-saline) was used to stain the surviving target cells.

Microcytotoxicity assay: experimental and theoretical considerations. Obtaining reproducible results in the microcytotoxic assay was found to depend on an optimal number of target cells in each well, so that valid comparisons could be made between control and test wells in each experiment. The optimal number of target cells is one which can be easily and reproducibly counted and sufficiently large so that "in-test" variability is a relatively small percent of mean number of cells in the control wells. In preliminary experiments to determine the optimum, 50, 100, 200, 500, and 1,000 target cells were planted in 12 wells of a microtiter plate, incubated for 48 h, and counted. Seeding 500 or more cells resulted in so many target cells that counting was difficult. Planting 100 cells or fewer yielded only about 9–18 cells/well because of the plating efficiency of our strains. Since this small number of target cells would require a higher percent killing to be detected, we chose to use 200 target cells in the standard microcytotoxicity assay.

One of our early concerns related to the plating efficiency of the synovial target cells under the conditions of the cytotoxic assay. The design of the assay permitted a determination of the actual plating efficiency of the target cells used in each experiment, and no differences were found between RA and NR target cells. Based on the mean plating efficiencies, 32–36 individual target cells were present and countable in each control well when 200 cells were planted.

Other investigators have studied various aggressor-to-target cell ratios, ranging from less than 1:1 to 5,000:1. In general, 10:1, 100:1, and 500:1 aggressor-to-target cell ratios have been used most often.

Initially aggressor-to-target cell ratios of 100:1 and 10:1 were chosen for the studies with leukocyte aggressor cells because these ratios were within the range usually used by others, and using two different leukocyte concentrations allowed an evaluation of possible dose-related effects.

It should be noted that the stated ratios of aggressor to target cells of 100:1 and 10:1 were based on the number of target cells seeded, a practice customary in cytotoxic assays. However, based on the determined plating efficiencies, the actual aggressor-to-target cell ratios were on the order of 600:1 and 60:1.

The lymphocytotoxic tests were set up after the results of the leukocyte cytotoxic tests were available and only 100:1 lymphocyte: target cell ratios were studied. In view of the usual differential count on whole leukocyte suspensions, the lymphocyte: target cell ratios were two to three times higher in the tests using separated lymphocytes than in those using suspensions of unseparated leukocytes.

Statistical methods. The design of each test run and the statistical appraisal of cytotoxicity were intended to account for the important technical variables that could influence the outcome. The location of disease or control aggressor cells in the microtiter wells was continually varied and was unknown to the individual who counted surviving cells, so that results of cell counts could not be unconsciously influenced. Mean cell counts, standard deviations, and standard errors of the six wells containing the same aggressor and target cells were calculated. The differences between the mean of control and test sets were analyzed by the t test (6). Percent cytotoxicity was also calculated, with the control set as 0% killing. 50 unselected, consecutive tests were plotted to show the relationship of percent killing to the calculated value of t (Fig. 1). Regression analysis demonstrated a close fit, with $r^2 = 0.94$.

We have chosen to express the bulk of the results reported here in terms of the t value rather than percent killing, since we believe the t test better accounts for variability from day to day and within a given day's run. However, it should be noted that the results would not be different in terms of comparisons between RA and NR target or aggressor cells if we had chosen to express the results in terms of percent killing. From the plot of the regression analysis (Fig. 1), it can be seen that 28% killing produced a t of 2.1 ($P < 0.05$) and 40% killing gave a t of 2.9 ($P < 0.01$).

Since the t value in any given cytotoxic assay reflected the extent of killing, the value for t could be treated as a "cytotoxic score." Thus individual t values were termed the cytotoxic score to avoid confusion with comparisons made between the results obtained in the different patient groups with the t test for two means.

Chi-square (χ^2) analysis was performed by Fisher's exact method, and linear regression analysis by the least squares method was calculated with a programmed calculator (HP-65, Hewlett-Packard Co., Palo Alto, Calif.).

[2] Person, D. A., and J. T. Sharp. Unpublished observations.

Mycoplasma isolation attempts. The target cells and extracellular fluids were tested for mycoplasmal contamination with pleuropneumonia-like organism agar plates without inhibitors. Incubation was at 36°C under both aerobic and anaerobic conditions. Most of the tissues from which the cell strains were derived were cultured for mycoplasmas upon receipt in the laboratory. All cultures were negative. In addition, several of the target cell strains were studied with radioactive nucleotides as a probe for viral or mycoplasmal nucleic acid, and none was found (4).

RESULTS

Leukocytotoxicity for HEL and AND target cells. HEL cells served as the target cell in 172 microcytotoxic tests. In 68 of these tests, RA leukocytes were used as aggressor cells, 35 at 100:1 and 33 at 10:1 ratios. In 61 tests, leukocytes from patients with other diseases were used as aggressor cells, 34 at 100:1 and 27 at 10:1 ratios, Normal persons' leukocytes were used in 43 tests, 22 at 100:1 and 21 at 10:1 ratios. The results are shown in Table II. Almost no cytotoxicity occurred in any test group, the mean cytotoxic scores all being close to zero. Tabulation of the data to show the percent of tests positive at increasing cytotoxic scores for each of the leukocyte donor groups demonstrated that only occasional leukocytes from any source were moderately cytotoxic for HEL cells, and the three groups were comparable in the frequency of cytotoxic reactions as determined by χ^2 analysis (Table III).

TABLE II
Leukocyte Cytotoxicity for Target Cells

Leukocyte source	Mean cytotoxic score ±1 SE: 100:1 ratio	Mean cytotoxic score ±1 SE: 10:1 ratio	Target cell
RA	0.91±0.14	0.19±0.18	HEL
Other	−0.10±0.20	−0.12±0.21	
Control	−0.42±0.19	0.03±0.22	
RA	1.87±0.24	0.70±0.15	NR
Other	1.48±0.20	0.53±0.14	
Control	0.98±0.19	0.11±0.18	
RA	2.39±0.14	1.01±0.12	RA
Other	1.92±0.18	0.63±0.13	
Control	1.25±0.17	0.36±0.13	

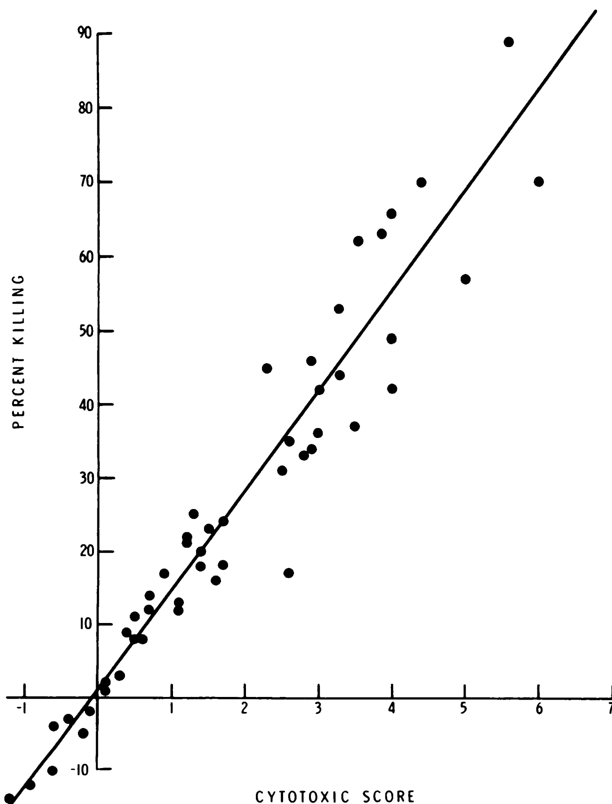

FIGURE 1 Linear regression analysis. The regression line is $y = 0.87 + 13.54\ x$ and $r^2 = 0.94$. Only 49 points appear in the figure as two are superimposed.

Assays carried out with the lupus skin fibroblast strain, AND, as the target cell gave similar results to the cytotoxic tests with HEL target cells. The AND cells served as the target cell in 13 assays with purified lymphocytes (9 RA and 4 normal). In no case did either the RA or control lymphocytes produce cytotoxic scores greater than 1.6 (mean cytotoxic score = 0.36).

Leukocytotoxicity for NR synovial target cells. A total of 430 tests was carried out with NR synovial target cells. There were 174 tests with RA leukocytes, 88 at 100:1 and 86 at 10:1 ratios, and 155 tests were carried out with other patients' leukocytes, 79 at 100:1 and 76 at 10:1 ratios. Normal persons' leukocytes were used in 101 tests, 51 at 100:1 and 50 at 10:1. The results of those tests demonstrated that leukocytes from

TABLE III
Cytotoxicity of Leukocytes for HEL Cells

Cytotoxic score	Leukocytes	Percent positive at stated cytotoxic score: 100:1	χ^{2*} (P)	10:1	χ^{2*} (P)
		%			
1.75	RA	6		12	
	Other	18		7	
	Control	9	2.6 (<0.3)	14	0.6 (<0.8)
2.12	RA	3		9	
	Other	6		7	
	Control	5	0.8 (<0.7)	0	1.9 (<0.4)
2.58	RA	0		0	
	Other	6		7	
	Control	0	3.4 (<0.2)	0	4.6 (<0.2)
2.92	RA	0		0	
	Other	0		0	
	Control	0	0	0	0

* χ^2 analysis was performed on each 2 × 3 contingency table.

TABLE IV

Cytotoxicity of Leukocytes for NR Synovial Target Cells

Cytotoxic score	Leukocytes	Percent positive at stated cytotoxic score			
		100:1	χ^{2*} (P)	10:1	χ^{2*} (P)
1.75	RA	44		20	
	Other	48		16	
	Control	29	4.7 (<0.1)	18	0.4 (<0.9)
2.12	RA	39		15	
	Other	35		11	
	Control	25	2.5 (<0.3)	12	0.8 (<0.7)
2.58	RA	33		12	
	Other	28		5	
	Control	18	3.8 (<0.2)	6	2.6 (<0.3)
2.92	RA	25		9	
	Other	22		4	
	Control	10	4.8 (<0.1)	4	2.5 (<0.3)

* χ^2 analysis was performed on each 2 × 3 contingency table.

all sources were more cytotoxic for NR synovial target cells than for HEL cells (Table II). At the 100:1 ratios, the differences between RA leukocytes and control leukocytes were significant, with t of 2.56 ($P <$ 0.02, df 137), and the differences between other leukocytes and control leukocytes were not significant, with t of 1.83 ($P > 0.05$, df 128). Differences between RA and other patients' leukocytes were not significant ($t =$ 1.24, $P > 0.2$, df 165). As with the HEL target cells, tests with NR target cells failed to detect significant differences between leukocytes from different patient groups (Table IV).

Lymphocytotoxicity: NR synovial target cells. NR synovial cells were used as target cells in 127 assays with lymphocytes (95 RA and 32 NR). The mean cytotoxicity of the RA lymphocytes (cytotoxic score = 0.78) did not differ significantly from that of the control lymphocytes (cytotoxic score = 1.01), with the t for the two mean cytotoxic scores being -0.63 ($P > 0.5$, df 125), and χ^2 analysis detected no differences (Table V).

TABLE V

Cytotoxicity of Lymphocytes for NR Synovial Cells

Cytotoxic score	Lymphocytes	Percent positive at stated cytotoxic score	
			χ^{2*} (P)
1.75	RA	27	
	Control	22	0.4 (<0.6)
2.12	RA	22	
	Control	13	1.4 (<0.3)
2.58	RA	20	
	Control	13	0.9 (<0.4)

* χ^2 analysis was performed on each 2 × 2 contingency table.

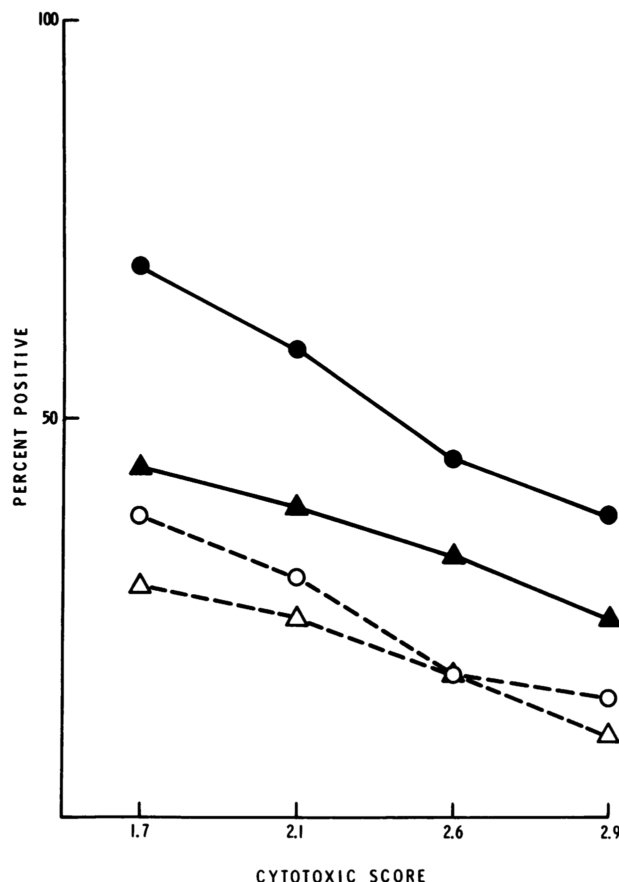

FIGURE 2 Leukocyte cytotoxicity for synovial cells. Closed symbols, RA aggressor cells; open symbols, control aggressor cells; circles, RA synovial target cells; triangles, NR synovial target cells. The aggressor-to-target cell ratios were all 100:1.

Leukocytotoxicity for RA synovial target cells. Synovial cells derived from patients with RA served as target cells in 789 tests. The leukocytes were from patients with RA in 386 tests, 197 at 100:1 and 189 at 10:1 ratios. Other patients' leukocytes were used in 234 tests, 123 at 100:1 and 111 at 10:1 ratios, and normal individuals' leukocytes were used in 169 tests, 87 at 100:1 and 82 at 10:1 ratios. In these tests RA leukocytes were highly cytotoxic for RA synovial cells (Table II). Leukocytes from patients with other diseases were cytotoxic less often, and normal individuals' leukocytes were cytotoxic least often. Differences in the cytotoxic scores of RA leukocytes versus other patients' leukocytes and RA leukocytes versus normal leukocytes, at the 100:1 ratios, were highly significant; RA versus other diseases, $t = 2.14$, ($P < 0.05$, df 318) and RA versus normal, $t = 4.8$, ($P < 0.001$, df 282). A plot of the percent of tests positive for RA and NR synovial target cells at increasing cytotoxic scores shows the striking cytotoxicity of RA leukocytes for RA synovial

TABLE VI

Cytotoxicity of Leukocytes for RA Synovial Target Cells

Cytotoxic score	Leukocytes	Percent positive at stated cytotoxic score 100:1	χ^{2*} (P)	10:1	χ^{2*} (P)
1.75	RA	69		34	
	Other	59		22	
	Control	38	23.4 (<0.0005)	13	14.0 (<0.001)
2.12	RA	59		24	
	Other	37		15	
	Control	30	25.9 (<0.0005)	5	15.5 (<0.0005)
2.58	RA	46		16	
	Other	33		8	
	Control	18	18.6 (<0.0005)	1	14.0 (<0.001)
2.92	RA	38		11	
	Other	28		5	
	Control	15	28.9 (<0.0005)	1	9.1 (<0.025)

* χ^2 analysis was performed on each 2 × 3 contingency table.

cells as contrasted to control leukocytes (Fig. 2). All comparisons at both the 100:1 and 10:1 ratios demonstrate significance by χ^2 analysis (Table VI).

In these tests the RA leukocytes were much more cytotoxic for RA synovial cells than for any other target cell. This is in contrast to the results obtained with other leukocytes. When individual results were analyzed, it was evident that the majority of highly cytotoxic reactions in the group of patients with other diseases occurred with leukocytes from patients with SLE and ankylosing spondylitis. Leukocytes from patients with gouty arthritis, degenerative joint disease, and osteopenia produced cytotoxic reactions similar to those obtained with normal individuals' leukocytes. Although leukocytes from patients with systemic lupus erythematosus and spondylitis were more cytotoxic for synovial cells than normal leukocytes, this cytotoxicity was not specific for either RA or NR target cells. These results were in contrast to those obtained with RA leukocytes (Table VII), which produced far greater killing of RA synovial target cells than NR synovial target cells. As indicated, leukocytes from NR individuals were cytotoxic in 25% of tests on NR target cells and in 30% of tests on RA target cells, whereas RA leukocytes were cytotoxic in 39% of tests with NR target cells compared to 59% of tests with RA target cells. The difference in the cytotoxicity between control and RA leukocytes for NR target cells was not significant (25% compared to 39%), whereas the difference in cytotoxic reactions between NR leukocytes (30%) and RA leukocytes (59%) for RA target cells was highly significant. Thus, RA leukocytes were more cytotoxic than NR leukocytes, and the cytotoxic reaction of RA leukocytes was directed preferentially against RA-derived synovial target cells.

Lymphocytotoxicity: RA synovial target cells. Purified lymphocytes from patients with RA were used in 159 tests and gave a mean cytotoxic score of 2.48±0.18 compared to 1.07±0.24 for the control lymphocytes used in 48 tests. The difference between mean cytotoxic scores was highly significant, $t = 4.09$, ($P < 0.001$, df 205). Tabulation of data at increasing scores confirmed

TABLE VII

Cytotoxic Reactions of Rheumatoid and Control Leukocytes for Synovial Cells

Leukocyte source	Target synovial cells NR	RA	χ^{2*} (P)
Control	13/51‡	26/87	0.3 (NS)
RA	34/88	116/197	10.0 (<0.005)
χ^{2*} (P)	2.5 (NS)	20.3 (<0.0005)	

* χ^2 analysis was performed on each 2 × 2 contingency table.
‡ Number with cytotoxic score ≥2.1/number tested.

TABLE VIII

Cytotoxicity of Lymphocytes for RA Synovial Cells

Cytotoxic score	Lymphocytes	Percent positive at stated cytotoxic score	χ^{2*} (P)
1.75	RA	54	
	Control	29	9.2 (<0.005)
2.12	RA	48	
	Control	19	12.9 (<0.0005)
2.58	RA	39	
	Control	15	9.9 (<0.005)
2.92	RA	34	
	Control	15	6.7 (<0.01)

* χ^2 analysis was performed on each 2 × 2 contingency table.

Table IX

Cytotoxicity of RA Lymphocytes for Synovial Cells

Cytotoxic score	Target cell	Percent positive at stated cytotoxic score	χ^{2*} (*P*)
1.75	RA	54	
	NR	27	17.2 (<0.0005)
2.12	RA	48	
	NR	22	16.6 (<0.0005)
2.58	RA	39	
	NR	20	9.9 (<0.005)
2.92	RA	34	
	NR	20	5.7 (<0.025)

* χ^2 analysis was performed on each 2 × 2 contingency table.

that RA lymphocytes killed RA synovial cells more than did control lymphocytes (Table VIII). A comparison of target cell data revealed that RA lymphocytes killed RA synovial target cells to a greater extent than NR synovial cells (Tables IX and X, Fig. 3).

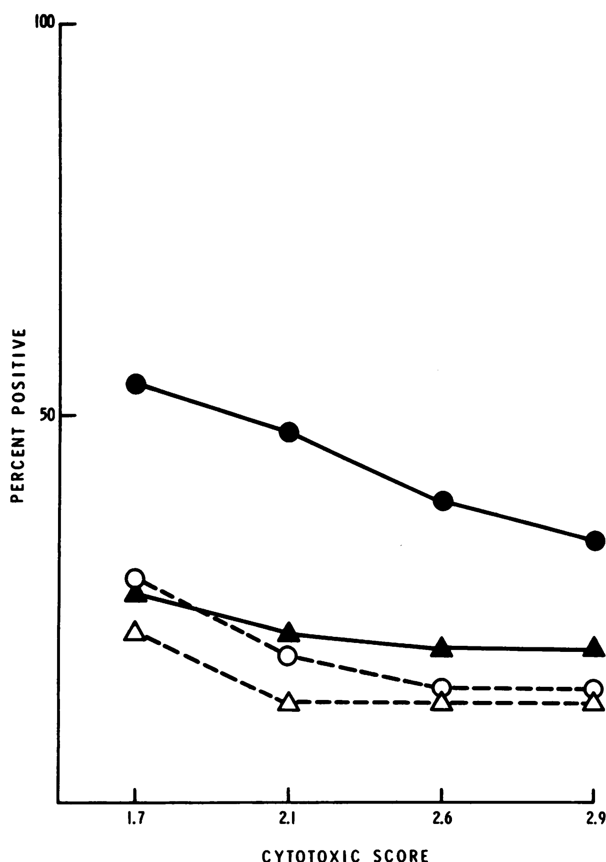

Figure 3 Lymphocyte cytotoxicity for synovial cells. Closed symbols, RA aggressor cells; open symbols, control aggressor cells; circles, RA synovial target cells; triangles, NR synovial target cells.

Table X

Cytotoxic Reactions of Rheumatoid and Nonrheumatoid Lymphocytes for Synovial Cells

Lymphocyte source	Target cell origin: NR	Target cell origin: RA	χ^{2*} (*P*)
Control	4/32‡	9/48	0.6 (NS)
RA	21/95	76/159	16.6 (<0.0005)
χ^{2*} (*P*)	1.4 (NS)	12.9 (<0.0005)	

* χ^2 analysis was performed on each 2 × 2 contingency table.
‡ Number with cytotoxic score ≥2.1/number tested.

To assess possible effects of "aging" of the target cells on the expression of cytotoxicity, multiple linear regression analyses were carried out with each combination of aggressor and target cells; namely, RA lymphocytes versus RA target cells, control lymphocytes versus RA target cells, RA lymphocytes versus NR target cells, and control lymphocytes versus NR target cells. The two variables used were cytotoxic scores and the passage level of the target cell (Table I). The calculated r^2 values varied from 0.01 to 0.11, indicating no relationship. These data are not consistent with the unmasking or loss of antigens(s) from target synovial cells with time in culture.

DISCUSSION

The most striking observation reported here is the frequent cytotoxicity of rheumatoid lymphocytes for synovial cells from patients with RA grown in vitro. To determine the significance of this observation, a number of technical features of the test system were evaluated and a number of comparisons of aggressor cells and target cells from various sources have been made. If, for example, the plating efficiencies of the RA and NR synovial cells had been significantly different, the results might have been explicable on that basis. Likewise, we specifically chose the HEL fibroblast cell strain as one of the target cells to evaluate nonspecific killing, since there was no reason to expect RA lymphocytes to be sensitized to a nonsynovial fibroblast of embryonic origin. In the study design, all tests were set up in parallel with HEL cells, NR synovial cells, and RA synovial cells as target cells with aggressor cells from each donor category, i.e., RA patients, other patients, and normal persons, in each day's run to provide an internal control. It is worth commenting that the cytotoxic score is a statistical comparison of test wells with target cell control wells that takes into account variation in the number of target cells in the sets of control and test wells in each day's run.

After the determination that RA leukocytes were cytotoxic for RA synovial cells, separation experiments demonstrated that cell suspensions containing 97–98% lymphocytes and less than 2.5% macrophages retained all of the net cytotoxicity for synovial cells. These results strongly suggest that the cytotoxic activity is largely a property of lymphocytes. It is not known whether the effector lymphocytes are bone marrow- or thymus-derived cells, nor whether cooperation of small numbers of macrophages is required.

In the cytotoxic tests reported here, synovial target cells from all sources were more frequently killed by both unseparated leukocytes and lymphocytes than were fibroblasts from other sources. Leukocytes from patients with CTD, in which immune mechanisms are thought to play a pathogenic role, were more cytotoxic for synovial cells than leukocytes from normal persons. However, cytotoxicity of CTD leukocytes was as great for NR as for RA synovial cells.

Leukocytes from patients with RA were cytotoxic for NR synovial cells at about the same frequency as leukocytes from all patients with CTD and other musculoskeletal conditions were cytotoxic for both NR and RA synovial cells. In striking contrast, RA leukocytes were far more cytotoxic for RA synovial cells than NR synovial cells. When purified lymphocytes were used as aggressor cells, a lower frequency of killing was observed, but the difference in frequency of cytotoxic reactions between RA and control aggressor cells, namely the net cytotoxicity, was similar for both leukocytes and lymphocytes when RA synovial target cells were used.

The unique susceptibility of RA synovial cells to the cytotoxic effects of RA leukocytes and lymphocytes is compatible with several possible explanations. First, immune recognition of an antigen in RA-derived synovial cells in culture could be responsible for the observed reactivity. Several candidates for the putative antigen might be considered, including viruses, mycoplasmas, other microorganisms, and nonliving antigens acquired from the environment. The synovial cells may be chronically infected with one of the aforementioned agents and the cytotoxic reaction may be directed against a microbial antigen. Alternatively, one of the agents may have induced a cellular membrane change recognized by sensitized RA lymphocytes. Virus-infected cells and virus-transformed cells can be recognized and killed by sensitized lymphocytes (7, 8). At least hypothetically, a mycoplasma or mycoplasmal-induced antigen might be detected by the cytotoxic reaction.

Alternatively, the unique reactivity of the RA synovial cells may reflect an endogenous tissue antigen, such as a disease-specific tissue antigen, a fetal synovial cell antigen derepressed by the disease process, or a histocompatibility (HLA) antigen. These antigens would presumably be inherited, but evidence to date does not suggest that RA is an inherited disease. In view of the reports of a normal distribution of HLA-A and B antigens in RA (9–12), it seems unlikely that an HLA-A or B antigen is involved, since if RA lymphocytes were responding to HLA antigens, then one would expect cytotoxic reactions as often with NR as RA synovial cells. Mixed lymphocyte culture reactivity, suggesting an unusual frequency of antigens at the HLA-D locus in patients with RA, has been reported (13). Further investigation is required to determine whether HLA-D antigen distribution has any bearing on the cytotoxic reactions observed. To our knowledge, no studies on the HLA-C locus in RA have been published. It should be noted that the possible participation of a histocompatibility antigen in determining the specificity of the cytotoxic reaction does not exclude the participation of a virus or viral induced neo-antigen. Studies in mice have shown that the cytotoxicity of sensitized lymphocytes for virus-infected cells and tumor cells is in part determined by the H-2 antigens (14).

Finally, the unique reactivity of RA synovial cells could be accounted for by the presence of a nonimmunologically specific stimulator in the synovial cells, unique to or present in greater amount in RA synovial cells. Although this latter possibility seems least likely, it cannot be discounted, because a recent report described the mitogenic activity of various blood components (15). Castor has clearly documented differences in the metabolic activity of RA synovial cells as compared to NR cells. Those differences related primarily to increased hyaluronate and lactate formation and glucose uptake by RA synovial cells. Increased concentrations of "connective tissue-activating peptide" have also been found in RA synovial cells (16, 17). However, none of these materials has been shown to stimulate lymphocytes.

That cytotoxicity did not occur in all tests with RA aggressor and RA synovial cells requires explanation. A number of laboratory, X-ray, and clinical findings were evaluated for possible relationships to the cytotoxic scores. None of the results was sufficiently striking to warrant extensive discussion, except to state that medication, seropositivity, disease activity, and duration of disease did not correlate with the degree of cytotoxicity. Perhaps only a minority of patients with RA are sensitized to the synovial antigen or are responsive to the synovial factor that initiates the cytotoxic reaction. On the other hand, the low frequency of cytotoxicity may reflect the sensitivity of the test. Low efficiency of killing could be due to a small amount of antigen on the target cell, a small number of sensitized cells in the host lymphocyte pool, or a low susceptibility to cell-mediated lysis on the part of RA synovial cells.

The results might also be interpreted as indicating that the RA synovial cells represent two or more populations. This might be because RA is a heterogenous

condition with more than a single etiology. Alternatively, the outgrowth of cells from the initial synovial tissue explants may be heterogenous, and cells carrying the inducing agent, presumably an antigen, may not be represented in all of the cell strains. If HLA antigens play some role in the reaction, one might anticipate that not all mixtures would have the right combination of sensitized aggressor cells and target cells, since only about 48–68% of patients with RA possess HLA-DW4 (13).

A recent abstract by Griffiths et al. suggested that the degree of cytotoxicity produced by RA or NR lymphocytes for synovial target cells was related to the age of the target cells in culture (18), an observation we were unable to confirm with the present data. A number of other investigators have studied the cytotoxic effects of both synovial fluid cells and peripheral white blood cells obtained from patients with CTD for a variety of target cells (18–26). None has made the quantitative comparisons between RA and NR synovial cells shown here, and because of many variables in the techniques used, their results have little bearing on the interpretation of the present observations.

As indicated above, a number of possibilities could account for the observations made. Possible nonspecific, metabolic, or genetic effects have been considered and remain as tenable explanations; however, the data reported strongly suggest the possibility that RA lymphocytes were sensitized to an antigen(s) present on RA synovial cells. Definition of the antigen(s) requires further investigation.

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