Immune Function in Severe, Active Rheumatoid Arthritis

A Relationship Between Peripheral Blood Mononuclear Cell Proliferation to Soluble Antigens and Synovial Tissue Immunohistologic Characteristics

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Abstract. The immunohistology of synovium from a tender, swollen knee and peripheral blood cellular immune function were correlated in 24 clinically similar patients with active, seropositive rheumatoid arthritis who were not taking cytotoxic or long-acting antirheumatic drugs. The patients were classified as anergic (n = 6) or nonanergic (n = 18) on the basis of peripheral blood mononuclear cell proliferative responses to a battery of soluble recall antigens. The peripheral blood mononuclear cells of anergic patients failed to respond significantly to any soluble recall antigen, whereas cells from nonanergic patients responded to at least one such antigen. Multiple pieces of synovial tissue were obtained from each patient at arthroscopy. To minimize intrajoint variability, all pieces were analyzed and averaged to determine a composite profile of abnormalities. Synovial specimens from all six anergic patients had "high intensity" lymphocytic infiltration (group A). In sharp contrast, synovial specimens from 15 of 18 nonanergic patients had "low intensity" lymphocytic infiltration (group B) (P = 0.002). Group A tissues typically showed higher intensity T cell and plasma cell infiltration, more synovial lining layer hyperplasia, more HLA-DR bearing cells, and a higher ratio of Leu 3A/Leu 2A T cells than did group B. Group B tissues had fewer infiltrating cells (most of which were OKM1 and HLA-DR bearing), more extensive fibrin deposition, and far fewer T and plasma cells. Although these data do not imply that synovium from different joints in an individual patient are immunohistologically identical, they do provide evidence that peripheral blood mononuclear cell immune function reflects immunopathologic events in the biopsied joint. Moreover, the data further support the view that clinically active rheumatoid arthritis is, like certain other chronic inflammatory conditions, a heterogeneous disorder with polar subgroups.

Received for publication 3 January 1984 and in revised form 11 June 1984.

The Journal of Clinical Investigation, Inc.
Volume 74, October 1984, 1173–1185

Introduction

Although the American Rheumatism Association criteria (1) for rheumatoid arthritis define a readily recognizable disorder, increasing evidence indicates that the condition is heterogeneous. It is well known that patients with active disease vary widely in age of onset, course, severity, outcome, and responses to therapeutic modalities. Immunologic parameters in active rheumatoid arthritis are also highly variable. For example, reports of T cell subset ratios ($OKT4^+$ or Leu $3A^+$ to $OKT8^+$ or Leu $2A^+$) in peripheral blood have varied from 0.9 to 7.5. In synovial fluid, T cell subset ratios ranging from 0.2 to 2.8 have been reported (2–7). Percentages of HLA-DR[1] bearing cells in these two compartments vary from 3.2 to 50% (2–9).

Functional differences have also been described. For example, we have studied the proliferative responses of peripheral blood mononuclear cells from clinically similar patients with active rheumatoid arthritis after stimulation in vitro with

1. *Abbreviations used in this paper:* H + E, hematoxylin and eosin; HLA-DR, nonpolymorphic DR antigens of the major histocompatibility locus antigen region; PTAH, phosphotungstic acid-hematoxylin.

various soluble recall antigens and observed a wide spectrum of responses, ranging from nonresponse to normal. It is interesting that the nonresponders to soluble antigens (anergic subgroup) clinically improved after a course of mononuclear cell removal by short-term repeated leukapheresis, with a significant, although transient, decrease in articular indices, whereas the nonanergic patients failed to improve significantly (9). These observations suggested that peripheral blood cellular immune function (as reflected by the status of in vitro peripheral blood mononuclear cell response to soluble antigens) and synovial tissue immunopathology are interrelated. The present report provides evidence that cellular immune function and the immunohistology of synovium from a clinically active knee of patients with active rheumatoid arthritis are, indeed, interrelated. Synovial biopsy specimens from an active knee joint of anergic patients were more heavily infiltrated with Leu 1^+, Leu 3^+ T lymphocytes, HLA-DR^+ cells, and plasma cells, and they showed more lining layer hyperplasia and less sublining layer fibrin deposition than did biopsy specimens from inflamed knee joints of nonanergic patients. The results support the view that rheumatoid arthritis is a heterogeneous disorder with polar subgroups, and that the heterogeneity is reflected in characteristic profiles of peripheral blood mononuclear cell immune function and synovial immunopathology.

Methods

Patients. All patients had definite or classical seropositive rheumatoid arthritis as defined by American Rheumatism Association criteria (1). None was taking drugs other than nonsteroidal antiinflammatory agents and/or prednisone (≤10 mg/d) at the time of biopsy, and all other cytotoxic and/or second line antirheumatic agents had been discontinued for at least 4 wk. All had active disease as defined by a Ritchie-Camp articular index (10, 11) of ≥40, with sustained disease activity during the 2 mo before study. The biopsied knee had been actively inflamed for at least 2 mo before biopsy, and in many cases longer. There was no difference in the duration of active knee inflammation in anergic and nonanergic patients. In no case was the current episode of knee inflammation the first for that joint. No patient had major medical disorders other than rheumatoid arthritis.

After patients gave informed consent, arthroscopically guided synovial biopsies were performed solely for research purposes under a protocol approved by the Clinical Research Committee of the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases at the Clinical Center of the National Institutes of Health.

Normal volunteers. Peripheral blood was obtained from 20 healthy normal volunteers (13 females and 7 males) who were taking no medications.

Synovial tissue analysis. 15 to 20 pieces (1–2 g) of synovium from a clinically active (tender and swollen) knee were obtained at arthroscopy by the use of a pea-pod otolaryngologic biopsy forceps. To minimize sampling error, pieces were taken randomly from the suprapatellar, medial, and lateral compartments of the joint. The tissue was put into sterile phosphate-buffered saline (PBS) and washed in several changes of sterile PBS. Each piece was then divided, and part was fixed in 10% phosphate-buffered formalin and part was snap frozen in a glycerol base embedding medium (O.C.T., Tissue-Tek II; Lab-Tek, Div. of Miles Laboratories, Inc., Naperville, IL) by immersion in a mixture of dry ice and acetone. Frozen blocks were stored at −20°C in sealed containers until sectioned for staining. 8-μm sections were cut on a cryostat at −20°C (model CTI; International Equipment Co., Damon Corp., Needham Heights, MA) and placed on glass slides. The slides were dipped into a 60°C solution of 0.5% wt/vol gelatin, 0.024% wt/vol chromium potassium phosphate, 0.015% wt/vol thymol in distilled water, and allowed to air dry before tissue was applied. Slices from all pieces of snap frozen tissue were included on all sections stained.

The sections were fixed in room temperature acetone for 5 min, and cellular antigens were demonstrated by the use of the monoclonal antibodies listed in Table I in combination with a sensitive immunoperoxidase staining technique (22, 23) (ABC Vecta Stain Kit; Vector Laboratories, Inc., Burlingame, CA). Color was developed by immersing the sections in a solution of 0.05% wt/vol 3,3,diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO), 0.04% wt/vol nickel chloride, and 0.003% hydrogen peroxide in 0.05 M Tris/0.15 M NaCl buffer, pH 7.4. Sections were counterstained with 2% methyl green in methanol, dehydrated in three changes of absolute ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ) under a glass coverslip. Control sections were stained with no primary antibody, or with mouse ascites fluid or normal mouse IgG.

Cell surface antigen grades and inflammatory index. In immunoperoxidase-stained sections, the percentage of stained cells for each antibody was estimated to the nearest 10% in all fields of the entire section under light microscopy. To account for the variation in numbers of infiltrating cells in different fields, each field was assigned a weight score of 1–10, reflecting very low to very heavy cellular infiltration. To arrive at a grade proportional to the number of positive cells per field bearing a particular cellular antigen, the following formula was used for each antigen: Grade = $\{\sum_{\text{all } n}$ [(percentage of cells stained in field n) × (weight score of field n)]$\}$/total number of fields scored.

For example, if all cells in all fields on a section were positively stained, the grade on that section for that surface marker would be 1,000 (maximum possible grade). Similarly, if no positive cells were seen anywhere in the section, the grade would be 0.

Formalin-fixed tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H + E), and phosphotungstic acid-hematoxylin (PTAH) by standard procedures. Coded H + E-stained sections were graded by an inflammatory index derived from an index employed by Cooper et al. (24). We assigned to each field a grade (inflammatory index) of 0–10 points, proportional to the intensity of infiltrating lymphocytes. For example, a field with dense and diffuse lymphocytic infiltration received a grade of 10 points, whereas a field with no lymphocytes received a grade of 0. Fibrin deposition, plasma cell infiltration, and synovial lining layer hyperplasia were each independently graded on a scale of 0–3 points in each field.

Multiple high-power fields (40 or more) in each section were graded, and the average grades for inflammatory index, plasma cell infiltrate, fibrin deposition, lining layer hyperplasia, and various cell surface antigen grades were calculated separately. The sections contained slices from all the tissue obtained at arthroscopy. The sections were scored independently by two of the authors (Drs. Malone and Tsokos) without knowledge of the peripheral blood mononuclear cell proliferative responses discussed below. Where interobserver variation exceeded

Table I. Monoclonal Antibodies Used in Immunoperoxidase Staining of Synovial Tissue

Antibody	Specificity	Source	References
Leu 1	Pan T cell	Becton-Dickinson, Monoclonal Antibody Center, Sunnyvale, CA	12
Leu 2A	T cytotoxic/suppressor cell	Becton-Dickinson, Monoclonal Antibody Center, Sunnyvale, CA	13, 14
Leu 3A	T helper/inducer cell, activated monocyte/macrophage	Becton-Dickinson, Monoclonal Antibody Center, Sunnyvale, CA	13–15
Leu 7	Natural killer/killer cells	Becton-Dickinson, Monoclonal Antibody Center, Sunnyvale, CA	16
Leu M1	Monocyte/macrophage	Becton-Dickinson, Monoclonal Antibody Center, Sunnyvale, CA	17
Leu M3	Monocyte/macrophage	Becton-Dickinson, Monoclonal Antibody Center, Sunnyvale, CA	18
Anti-HLA-DR	HLA-DR nonpolymorphic antigen	Becton-Dickinson, Monoclonal Antibody Center, Sunnyvale, CA	19
OKM1	Monocyte/macrophage, some natural killer/killer cells	Ortho Pharmaceutical, Raritan, NJ	20
63D3	Monocyte/macrophage	Coulter Electronics, Inc., Hialeah, FL	21

20%, all fields on the slide were jointly reviewed and a final average score was agreed upon. This was necessary in ~20% of specimens, but in no case did review result in a change in group classification of a patient.

Peripheral blood mononuclear cell proliferative responses. Peripheral blood mononuclear cell proliferative responses to a battery of soluble recall antigens in vitro were determined as previously described in detail (9). In brief, peripheral blood mononuclear cell incorporation of tritiated thymidine was measured in vitro after stimulation by several soluble recall antigens. Antigenic stimuli included purified protein derivative (at 1, 2 and 5 μg/ml of culture medium, Connaught Laboratories, Toronto); streptokinase-streptodornase (at 1, 2, and 5 U/ml) and tetanus toxoid (at 1, 2 and 5 Lf/ml, Lederle Labs Div., American Cyanamid Co., Pearl River, NY); and candida (diluted to final concentrations of 1:1,000, 1:2,000, and 1:10,000 of the original stock solution as supplied by the manufacturer, Hollister-Steir Laboratories, Spokane, WA). These doses included the previously determined optimal dose as well as a higher and lower concentration to detect shifts in dose response. Cultures were incubated for 6 d and, after a 4-h pulse with 0.5 μCi/ml tritiated thymidine (specific activity 6 Ci/mM, Schwarz/Mann, Div., Becton Dickinson Immunodiagnostics, Orangeburg, NY), the cells were harvested and processed for the determination of tritiated thymidine incorporation. A stimulation index was calculated by dividing the mean tritiated thymidine incorporation of triplicate antigen-stimulated cultures by the mean tritiated thymidine incorporation of triplicate unstimulated cultures. Orally administered corticosteroids were withheld for at least 24 h before peripheral blood for proliferation studies was taken, and these blood samples were obtained within 7 d of synovial biopsy.

Statistical analysis. The means of the various synovial tissue characteristics in Table III were compared by use of the Student's *t* test, employing the unequal variance model where necessary, and associations in Fig. 2 were tested by Fisher's exact test (25, 26).

Results

Peripheral blood cellular immune status and clinical profiles of patients. Consistent with our previous experience, peripheral blood mononuclear cells from normal healthy volunteers almost without exception had stimulation indices of >2 to at least one soluble recall antigen (Fig. 1) (9). Based on this criterion, we categorized our rheumatoid arthritis patients into two subgroups, anergic and nonanergic (9). As shown in Fig.

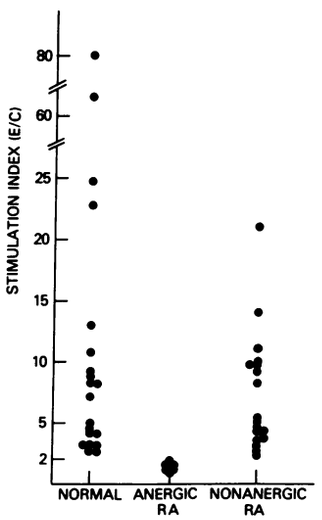

Figure 1. Stimulation indices of normal volunteers and patients with rheumatoid arthritis. Each point represents an individual's maximum stimulation index to one of the following soluble antigens: purified protein derivative, tetanus toxoid, streptokinase-streptodornase, or candida. The stimulation index is the tritiated thymidine incorporation (counts per minute) of antigen-stimulated mononuclear cells divided by the tritiated thymidine incorporation of unstimulated (control) cells. An anergic response is defined as a stimulation index of ≤2 for all antigens tested, and a nonanergic response is defined as a stimulation index of >2 to one or more antigens tested. E/C, experimental/control.

1, peripheral blood mononuclear cells from 6 patients were anergic (stimulation indices of ≤2 to all soluble recall antigens tested on two or more occasions), and cells from 18 patients were nonanergic (stimulation indices of >2 to one or more antigens). There were no significant differences in the level of spontaneous or unstimulated thymidine incorporation between the anergic and nonanergic subgroups (2,909±790 cpm for anergic patients and 3,520±473 cpm for nonanergic patients). Absolute counts of peripheral blood mononuclear cells (2,048±809 cells/mm^3 for anergic patients and 1,615±695 cells/mm^3 for nonanergic patients) were similar in both groups. Moreover, there were no differences between anergic and nonanergic patients in past and present medication regimens, duration of disease, Ritchie-Camp articular indices, Ritchie-Camp indices of the biopsied joint, or erythrocyte sedimentation rates (Table II). Nor were there differences in the number of active joints, duration of morning stiffness, presence of rheumatoid nodules, presence of erosive radiographic changes, history of rheumatoid lung disease (one patient in the anergic, high intensity group and one patient in the nonanergic, low intensity group), or bentonite flocculation titer (data not shown). No patient had a history of rheumatoid vasculitis, episcleritis, or Felty's syndrome.

Knee synovial fluid was obtained at arthroscopy in seven patients (five nonanergic and two anergic) in amounts varying from 0.5 to 75 cc. Synovial fluid white blood cell counts varied from 3,700 to 70,400, with polymorphonuclear leukocytes predominating (range 68–96%), and with lymphocytes (range 1–22%) and histiocytes (range 1–10%). There were no apparent differences in cell counts, differentials, or chemical analysis in fluids from anergic and nonanergic patients.

Thus, initially, the sole basis for the segregation of the patient population into two subgroups was the ability of their peripheral blood mononuclear cells to respond to soluble recall antigens in vitro. It should be noted from previous studies that responses to mitogenic lectins were similar in the patient subgroups (9).

Synovial tissue inflammatory indices and cellular immune

Table II. Historical and Clinical Patient Data

Patient	Disease duration	Ritchie-Camp articular index	Ritchie-Camp score* of biopsied joint	Sedimentation rate	Previous drugs‡	Proliferation§
	yr			*mm/h*		
EJ	16	87	3/3	27	G, C, N	AN
LL	15	50	2/2	71	G, *d*P, A, N	AN
SS	4	50	1/3	128	G, *d*P, N	AN
RBa	7	93	2/3	70	G, *d*P, P, A, N	AN
JK	9	55	2/3	120	G, *d*P, A, N	AN
DM	17	72	3/3	42	G, *d*P, P, A, N	AN
AM	9	70	3/3	120	G, C, N	N
WD	18	60	3/3	76	G, P, N	N
PS	12	71	3/3	48	G, *d*P, N, MTX	N
MJ	15	80	3/3	123	G, *d*P, A, N, Cy	N
AQ	16	50	3/3	81	G, *d*P, P, A, N	N
DG	9	91	1/3	70	G, *d*P, N	N
ACr	2	60	2/2	57	G, *d*P, N, MTX	N
IA	10	43	1/1	124	G, *d*P, P. N, MTX	N
TD	36	73	3/3	95	G, *d*P, P, N	N
TL	16	62	2/3	72	G, *d*P, P, A, Ph, N	N
LB	25	44	1/3	85	G, *d*P, P, N	N
JD	17	49	3/3	92	G, P, A, N	N
RJ	5	50	3/3	82	Ph, N	N
RS	3	66	3/3	50	P, Ph, N	N
MB	9	90	3/3	91	G, *d*P, C, A, N	N
AT	6	55	2/2	105	G, *d*P, C, A, N	N
AC	7	95	3/2	70	G, *d*P, C, A, N	N
RBr	8	75	3/3	118	G, *d*P, C, A, N, MTX	N

* Tenderness (0–3 points)/swelling (0–3 points). ‡ A, azathioprine; C, chloroquine; Cy, cyclophosphamide; *d*P, *d*-penicillamine; G, gold salts; MTX, methotrexate; P, hydroxychloroquine; N, nonsteroidal antiinflammatory agents; Ph, phenylbutazone. § AN, anergic; N, nonanergic.

status. To determine whether differences existed in the synovitis of these two subsets of rheumatoid arthritis patients, synovial biopsy specimens from clinically active knee joints were obtained and analyzed. Wide patient-to-patient variation in the average intensity of lymphocytic infiltration in the synovial biopsy specimens was observed (Fig. 2). Although few biopsy specimens showed uniform histology throughout (Table II), sampling and analyzing multiple sites allowed us to obtain a representative profile of the immunopathology in that joint. By multiple criteria, the overall composite features of tissue from anergic and nonanergic patients were clearly different. All 6 of the anergic patients had average synovial tissue inflammatory indices of >4 ("high intensity," or group A), whereas 15 of 18 nonanergic patients had average indices ≤4 ("low intensity," or group B). The association between high inflammatory index and anergy and low inflammatory index and nonanergic immune status was statistically significant by Fisher's exact test ($P = 0.002$). Viewed another way, the mean inflammatory index of anergic patients (5.3) was significantly higher than the mean inflammatory index of nonanergic patients (2.7, $P < 0.001$).

Synovial immunohistology. More extensive analysis of groups A and B biopsies by H + E and immunoperoxidase staining for cell surface markers is shown in Table III. By several of these criteria, the two groups were significantly different. Group A biopsies had a higher Leu 1 grade than did group B (37.7 vs. 7.2, $P < 0.005$) and had many more microscopic fields infiltrated with plasma cells and with mononuclear leukocytes in a nodular arrangement (Fig. 3 *A*). A large percentage of the infiltrating mononuclear leukocytes in the nodules expressed the pan T cell marker Leu 1 (Fig. 3 *B*). In such nodules, the ratio of Leu 3A-bearing cells to Leu 2A-bearing cells was as high as 10:1 (Fig. 3, *C* and *D*), and the Leu 3A-bearing cells were arranged in clusters. Leu 2A-bearing cells were more diffusely scattered. Leu 1-bearing cells were found elsewhere as well, in clusters around small sublining layer blood vessels and diffusely scattered in other areas without clear predominance of Leu 3A- or Leu 2A-bearing cells. Leu 1^+ cells were rarely found in the lining layer. The ratio of mean Leu 3A grade to mean Leu 2A grade in the group A tissues was 2.7, significantly higher than in group B, 1.0 ($P < 0.001$). One group A patient (LL) had a low Leu 1 grade compared with other group A patients. This is explained by the high plasma cell grade, indicating that most of the infiltrating lymphocytes in LL's tissue were plasma cells. Dense staining for OKM1 and HLA-DR antigens was seen on lining layer cells and on cells scattered in the sublining layer (Fig. 4, *A* and *B*). Similar staining was seen for 63D3, Leu M1, and Leu M3 antigens (not shown).

Although the intensity of HLA-DR staining varied considerably between specimens, the mean HLA-DR grade (proportional to the number of HLA-DR-bearing cells per field) was higher in group A (223.7) than in group B (70.7) ($P < 0.05$). The grades for lining layer hyperplasia were higher in group A (1.1) than in group B (0.4), ($P < 0.01$), as were the scores for plasma cell infiltration (1.4 vs. 0.4, $P < 0.001$). Leu 7-bearing cells were rare (<1%) in all tissues. Activated cells of monocyte/macrophage lineage in the lining layer bearing OKM1, HLA-DR, 63D3, Leu M1, and Leu M3 antigens also demonstrated faint intracytoplasmic staining for Leu 3A antigen, as previously reported in skin, peripheral blood, lymph node, and spleen (15). Small numbers (<5%) of cells in the nodular aggregates stained for OKM1, 63D3, Leu M1, and Leu M3, and somewhat larger numbers (0–20%) stained weakly for HLA-DR (Fig. 5, *A* and *B*).

In contrast, the overall composite features of group B biopsies were characterized by infiltration with far fewer cells, little or no lining layer hyperplasia, and, in many cases, a relatively homogeneous matrix which stained pink with H + E and blue with PTAH, thereby identifying fibrin as a major component (Fig. 6). Fibrin was also found in group A tissues, but it tended to be in a thin, ribbonlike distribution on the joint cavity side of the lining layer, or sparsely scattered in the sublining layer. Group B tissues had more fibrin than did group A tissues ($P < 0.05$). Leu 1-, Leu 2A-, and Leu 3A-bearing cells were rare, as were plasma cells. A large percentage of the cells present in these relatively hypocellular tissues bore the OKM1, 63D3, Leu M1, and Leu M3 markers, and many bore HLA-DR antigen as well (Fig. 7).

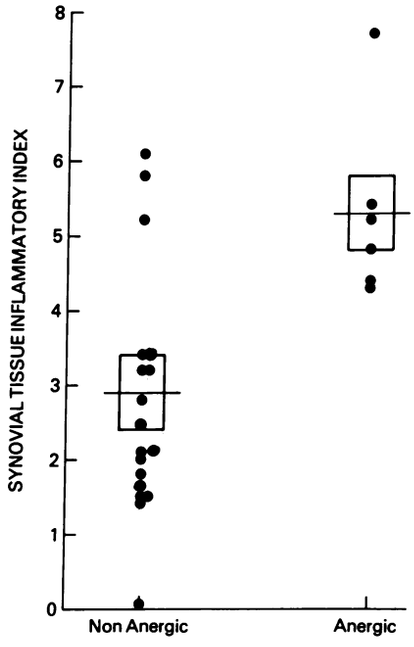

Figure 2. Inflammatory indices of synovial tissue specimens from anergic vs. nonanergic rheumatoid arthritis (RA) patients. The means and standard error of means are indicated by horizontal bars and boxes, respectively.

Table III. Synovial Tissue Characteristics

Patient	Inflammatory index (SEM)	Av Leu 1 grade	Ratio: Av Leu 3A grade / Av Leu 2A grade	Av HLA-DR grade	Av OKM1 grade	Lining layer thickness grade	Plasma cell grade	Fibrin grade
Group A								
EJ	7.7 (0.6)	71.9	3.4	542.2	113.3	2.1	0.5	0.0
AM	6.1 (0.4)	68.2	2.3	74.0	37.3	1.4	1.8	0.1
WD	5.8 (0.4)	52.6	1.3	277.9	157.8	1.3	0.4	1.5
LL	5.4 (0.5)	5.0	3.5	91.0	2.6	ND	2.3	1.0
SS	5.2 (0.3)	ND	ND	ND	ND	0.5	2.1	0.0
PS	5.2 (0.4)	26.1	2.7	86.3	42.4	0.5	1.8	1.2
RBa	4.8 (0.8)	22.2	1.7	353.1	26.2	1.0	1.3	0.9
JK	4.4 (0.9)	26.4	3.9	84.8	47.8	0.5	1.7	0.0
DM	4.3 (1.1)	28.8	2.6	280.6	248.6	1.3	1.0	2.1
Mean (SEM)		37.7 (8.4)	2.7 (0.3)	223.7 (60.1)	84.5 (29.5)	1.1 (0.2)	1.4 (0.2)	0.8 (0.3)
Group B								
MJ	3.4 (1.0)	4.0	1.0	39.4	59.5	0.5	1.1	1.1
AQ	3.4 (0.8)	13.5	0.8	88.6	7.2	1.0	0.3	0.7
DG	3.2 (0.8)	21.9	1.1	77.5	30.9	1.0	0.9	1.5
ACr	3.2 (0.5)	20.9	3.3	109.8	86.0	1.4	1.4	0.1
IA	2.8 (0.4)	1.6	1.7	120.8	56.8	0.6	0.0	1.0
TD	2.4 (0.7)	3.5	1.0	52.1	51.3	1.0	1.5	2.3
TL	2.1 (0.2)	12.9	0.9	72.9	48.8	0.1	0.7	0.8
LB	2.1 (0.4)	2.2	1.1	71.0	80.0	0.3	0.0	2.3
JD	2.0 (0.1)	0.3	0.0	22.5	0.1	0.0	0.0	0.7
RJ	1.8 (0.7)	3.3	1.1	117.7	60.0	0.0	0.5	2.5
RS	1.6 (0.2)	13.3	1.0	66.5	49.0	0.2	1.0	1.3
MB	1.5 (0.6)	14.0	1.5	48.8	106.6	0.2	0.2	2.5
AT	1.5 (0.4)	0.0	0.0	19.6	1.0	0.1	0.0	1.0
AC	1.4 (0.9)	4.2	1.0	83.0	75.0	0.2	0.1	0.2
RBr	0.0 (0.0)	0.9	0.0	49.8	49.8	0.0	0.0	3.0
Mean (SEM)		7.2 (1.9)	1.0 (0.2)	70.7 (8.1)	51.1 (8.0)	0.4 (0.1)	0.4 (0.1)	1.4 (0.2)
P value A vs. B		<0.005	<0.001	<0.05	NS	<0.01	<0.001	<0.05

Av, average; ND, not done; NS, not significant.

Discussion

We have provided evidence in the present study that synovial biopsy specimens from active knee joints of anergic rheumatoid arthritis patients are more heavily infiltrated by Leu 1^+/Leu $3A^+$ T lymphocytes, HLA-DR^+ cells, and plasma cells, and show more lining layer hyperplasia and less sublining layer fibrin deposition than do synovial biopsy specimens from clinically similar knee joints of nonanergic rheumatoid arthritis patients.

The identification of this relationship was critically dependent upon our methodology. We studied peripheral blood mononuclear cell proliferative responses, as reflected by tritiated thymidine incorporation in vitro, after stimulation of the cells with a battery of soluble recall antigens. The assays were repeated on two or more separate occasions, and no corticosteroids were given in the 24 h before the studies. This allowed us to reproducibly segregate our patients into anergic and nonanergic subgroups. Although skin test reactivity usually parallels the in vitro response, we have noted in previous studies that some patients, particularly those taking low-dosage steroids, have equivocal skin test reactivity but normal in vitro proliferative responses (9). In vitro peripheral blood mononuclear cell proliferation studies have proved to be more easily quantified and more reproducible than skin testing, and reliance on skin testing alone for characterization of anergic or nonanergic status probably would have resulted in more scatter in our data.

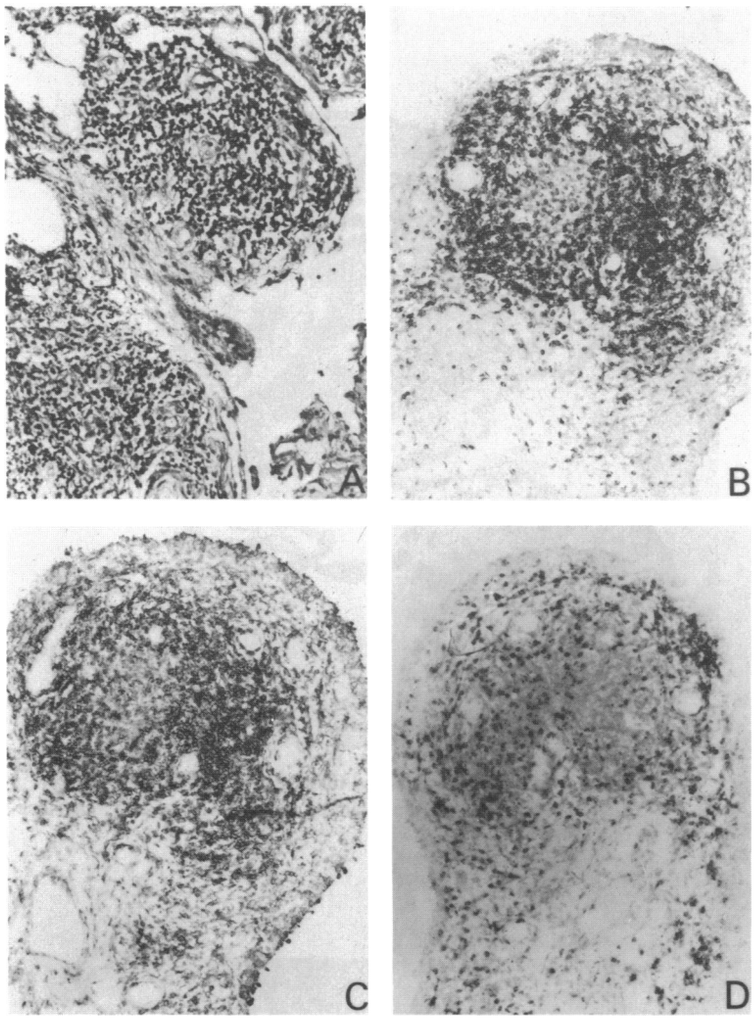

Figure 3. (*A*) H + E stain of a representative synovial specimen from a group A patient, showing two nodular aggregates of mononuclear cells. Original magnification, 220. (*B*) Immunoperoxidase stain of a mononuclear cell nodular aggregate in a representative synovial specimen from a group A patient, using the Leu 1 antibody. Black-ringed cells are positive for the Leu 1 antigen. Original magnification, 130. (*C*) Immunoperoxidase stain of a serial section of the same specimen as in *B*, using Leu 3A antibody. Note that most of the cells in the nodule bear the Leu 3A antigen. Original magnification, 130. (*D*) Immunoperoxidase stain of a serial section of the same specimen as in *B* and *C*, using the Leu 2A antibody. Leu 2A positive cells are much more scarce and scattered than are the Leu 3A positive cells. Original magnification, 130.

Our methods of obtaining tissue and our criteria for patient selection also need emphasis. None of our samples was obtained from patients on long-acting antirheumatic drugs or from patients undergoing joint replacement, as is typical of most published studies. Although most of our patients had been off such drugs for several months, a few (equally distributed between subgroups) had discontinued them as recently as 4 wk before biopsy. All of the patients in our study had severe, active polyarthritis, and withholding remission-inducing therapy for more than 4 wk was not considered justified. In addition, other investigators have reported histologic variation in synovial samples from the same joint (27). To minimize the possibility of obtaining nonrepresentative synovial tissue, in contrast to studies using needle biopsy, we obtained multiple pieces of tissue under direct visualization at arthroscopy from all areas of the knee joint accessible for biopsy through the arthroscope. All pieces obtained were represented on sections scored for various indices and grades. Thus, the immunohistologic assessment represented a composite or average of all tissue obtained from an individual patient. This approach allowed us to obtain a representative picture of the immunohistologic events in the biopsied joint and subsequently to categorize the synovial specimens.

Our study does not address the question of to what degree the synovium from the joint biopsied is comparable to that from other joints. We are interested in clarifying this question but cannot definitively do so at present, since it would require simultaneous biopsies of multiple joints in the same patient. Arthroscopy with synovial biopsy is an invasive procedure and, in our study, was done solely for research purposes. Protocol approval in the present study was given for biopsy of only one actively inflamed joint per patient. In any case, the data in this study do not imply that synovia from different joints in an individual are immunohistologically identical.

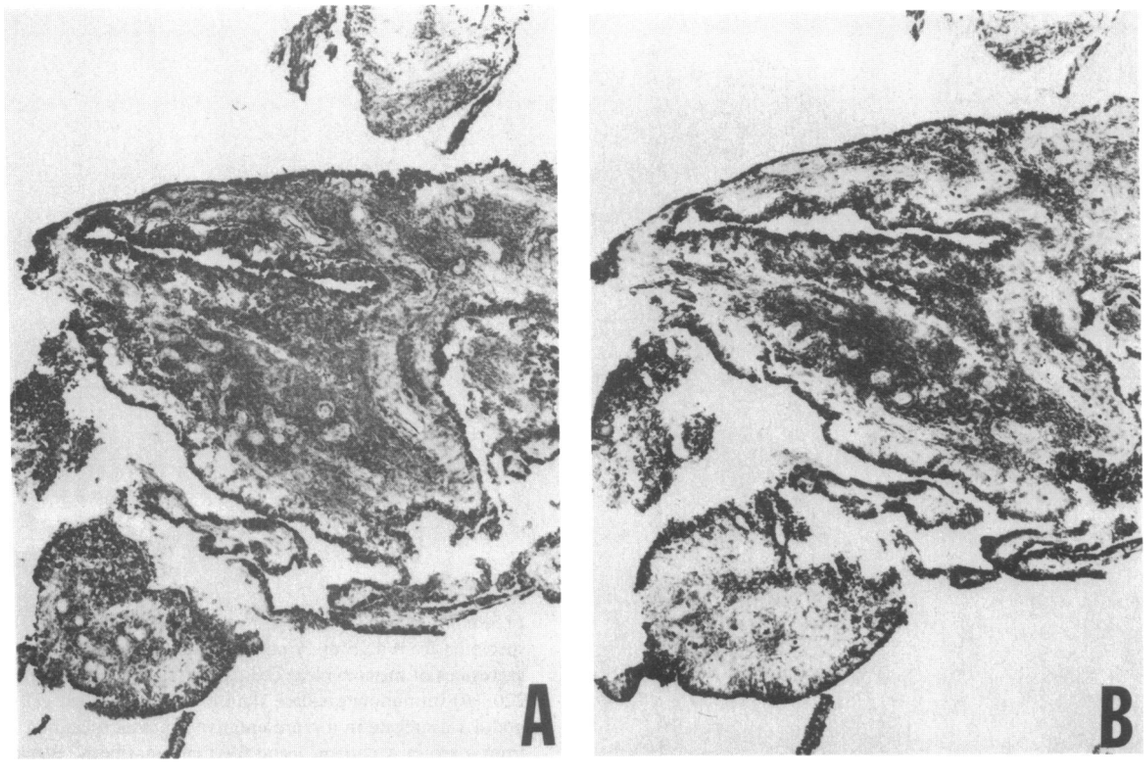

Figure 4. Immunoperoxidase-stained serial sections of a representative synovial specimen from a group A patient, using anti-HLA-DR antibody (*A*) and OKM1 antibody (*B*). Note the dense lining layer staining with both reagents and the presence of HLA-DR and OKM1-bearing cells in the sublining area as well. Original magnification, 54.

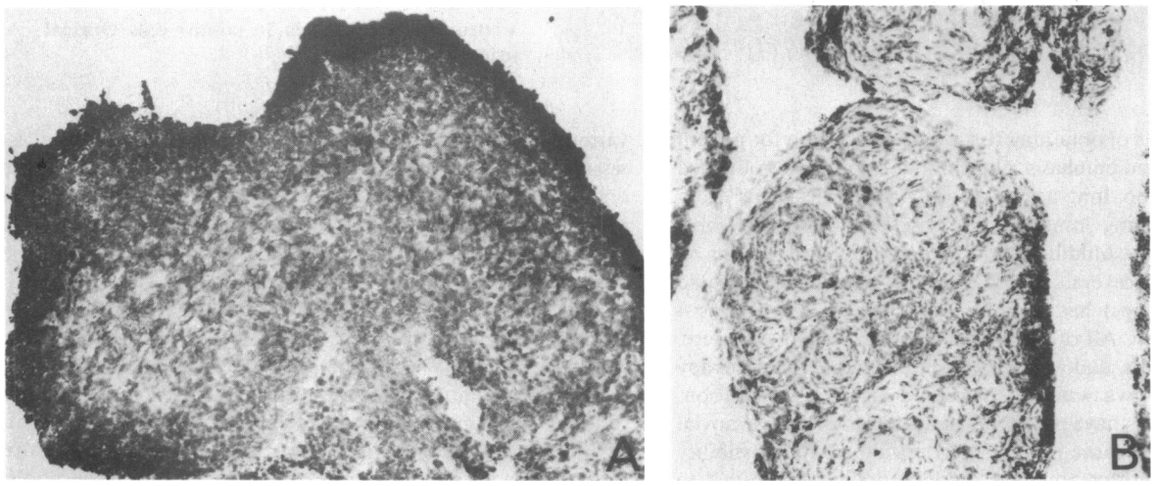

Figure 5. Immunoperoxidase-stained sections of a representative synovial specimen from a group A patient, showing a nodular aggregate stained with anti-HLA-DR antibody (*A*) and with OKM1 antibody (*B*). The section in *A* is notable for dense staining of lining layer cells, and weak staining of some of the cells in the nodular aggregate by anti-HLA-DR antibody. In *B*, the lining layer cells are densely stained, but very few of the cells in the aggregate bear OKM1 antigen. Original magnification, 130.

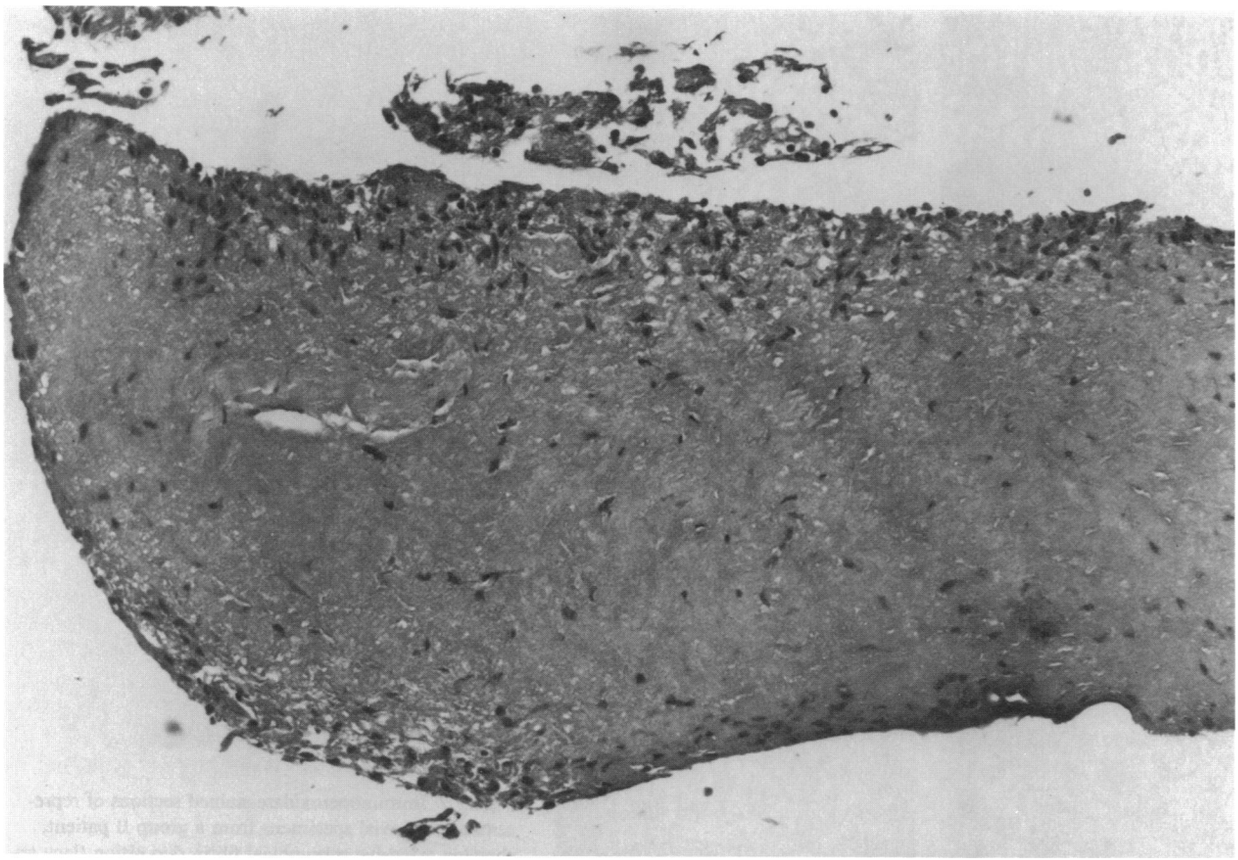

Figure 6. H + E-stained section of a representative synovial specimen from a group B patient, demonstrating a paucity of infiltrating cells, a thin lining layer, and a relatively homogeneous matrix. The matrix stained blue with PTAH, identifying it as fibrin. Original magnification, 130.

Indeed, we would expect some variation in the histologic appearance of synovium from different joints in the same patient, since their blood supply, lymphatic drainage, mechanical stresses, intraarticular pressures, and probably kinetics of mononuclear cell migration all differ. We would, however, anticipate that the same general associations demonstrated in the current study would be observed if active joints other than knees were compared. Specifically, we would expect synovial tissues from most active joints in the anergic subgroup to exhibit high intensity lymphocytic infiltration, whereas, on the other hand, we would expect wider joint-to-joint variation of inflammatory intensity in the nonanergic subgroup, since more variation existed between their knee biopsy specimens. The resolution of these issues does not bear directly on the validity of our current study and will need additional independent investigation.

The histologic abnormalities noted in the group A, or "high intensity," synovial tissues were consistent with previously published observations (28). Although not specific for rheumatoid arthritis, they included synovial lining layer hyperplasia; diffuse, nodular, and perivascular mononuclear cell infiltration in the sub-lining layer; fibrin deposition on the lining layer surface; and germinal center formation.

The data from immunoperoxidase staining of group A tissues confirm and extend the findings of other investigators who have used similar techniques to study rheumatoid arthritis synovium, in most cases obtained from knee joints (29–36). The studies have demonstrated that the tissue contains an abundance of HLA-DR-bearing cells. In addition, the aggregates of mononuclear cells are made up mainly of T lymphocytes, most of which bear the Leu 1 and Leu 3A surface markers. Although some cells with monocyte/macrophage markers are seen in the mononuclear cell aggregates, cells with these phenotypes are much more numerous in the lining layer and sublining layer regions. It is interesting that Leu 7^+ cells (natural killer cells) were rare.

In contrast to the group A synovial tissues, little attention has been given to the synovial tissues that we term group B,

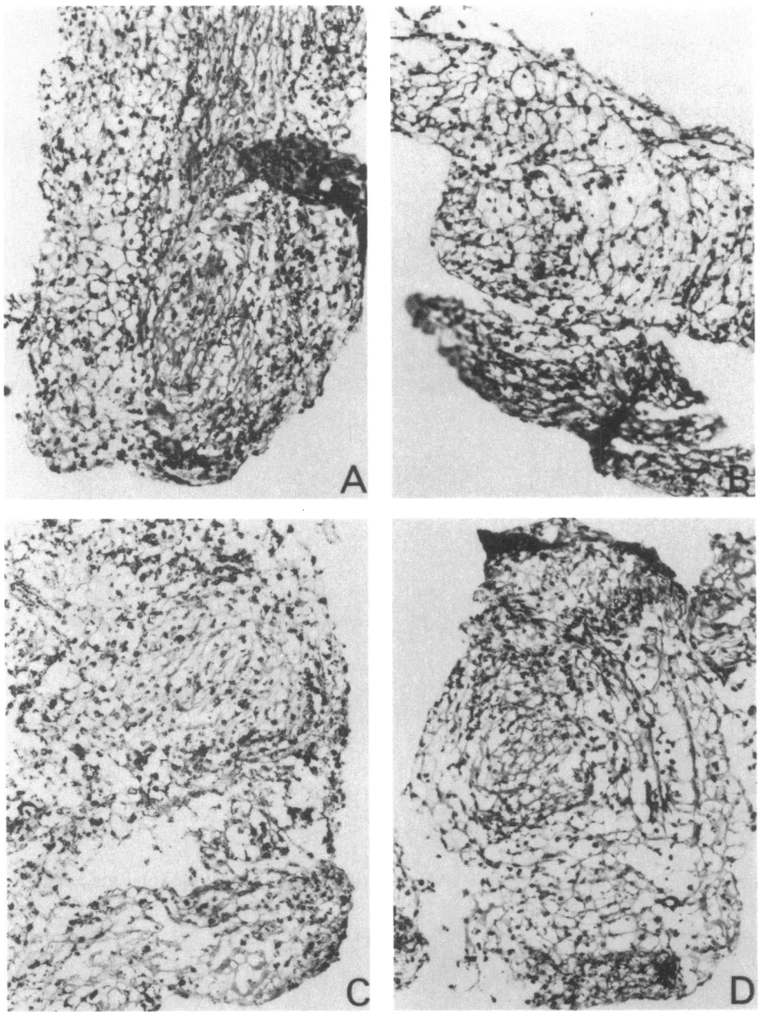

Figure 7. Immunoperoxidase-stained sections of representative synovial specimens from a group B patient, showing extensive subsynovial fibrin deposition (lacy appearing material on frozen sections) and demonstrating that a large fraction of the cells in such tissue are HLA-DR bearing cells of monocyte/macrophage origin. Tissue stained using: (*A*) anti-HLA-DR antibody, (*B*) anti-OKM1 antibody, (*C*) anti-Leu M1 antibody, and (*D*) anti-63D3 antibody. Original magnifications, 130.

or "low intensity," which are characterized by a paucity of total cells, rare T and plasma cells (most cells being of monocyte/macrophage lineage), a thin lining layer, and extensive fibrin deposition. No immunohistologic analysis of this type of synovium has been published. In 1951 Cruickshank observed a spectrum of synovial histology ranging from "fibrosis" to tissues heavily infiltrated with mononuclear cells. No quantification of the findings was given, however (27). Since then, several investigators have studied various properties of rheumatoid arthritis synovium and synovial cells and have noted fibrin deposition. None has quantified the extent of fibrin deposition or described these tissues in detail. Moreover, most have excluded up to 50% of their original collection of specimens from analysis because the tissues were "fibrotic," or lacked the classically described round cell infiltrates typical of our high intensity specimens (29, 37–45). We are aware of only one publication in which the authors suggest that immunohistologic study of rheumatoid arthritis synovium might be useful in identifying subsets of patients (30), but we have found no published reports in which this was actually done.

The immunohistologic profile we have demonstrated in rheumatoid synovium in many ways parallels that of a cell-mediated immune response (46, 47). Within the first 48 h of the tuberculin skin reaction, Leu 1^+ cellular aggregates appear and Leu $3A^+$ cells (instrumental in driving the early phase of the response [48]) are found in dense clusters and far outnumber Leu $2A^+$ cells, whereas the latter are scattered at low density throughout the infiltrates. Macrophages make up only a small fraction of the infiltrating cells at this early stage and are found mainly at the periphery of the nodule (49). As the cell-mediated immune reaction progresses, the number of cells bearing cytotoxic/suppressor phenotypes increases, and such

cells eventually predominate over cells bearing helper/inducer phenotypes. Macrophages appear in increasing numbers, and fibrosis eventually results.

In a recent study, Kurosaka and Ziff reported that HLA-DR staining was weak among cells of the mononuclear cell nodules, and dense on macrophages of the "transitional area"—the region at the nodule periphery that is populated by more macrophages, fewer T cells, a helper/suppressor ratio much lower than in the nodule, and by blastoid-appearing $OKT8^+$ cells. Their interpretation was that the perivascular nodule was a "quiescent" area, whereas the "transitional area" was immunologically active (50). In view of the similarities between the delayed type hypersensitivity reaction and the nodular lesions in synovium, we suggest that the nodules are acute immunologically active areas in which Leu $3A^+$ cells are reacting to a recent deposition of some as yet unknown stimulus or stimuli. The "transitional areas," by this construction, may represent later stages of granulation and repair processes. Weak HLA-DR expression on cells of the nodule does not imply immunologic quiescence of these cells (49).

It appears reasonable, therefore, to consider our group A tissues as representing a recent exacerbation of a characteristically waxing and waning cellular immune mediated inflammatory process, which, perhaps, is analogous to the early phases of the tuberculin reaction. That is, "high intensity" synovial pathology may reflect the host response to a new deposition of a causative stimulus or stimuli on a background of the more mature chronic inflammatory process. Group B tissues may represent the later-evolving reparative stage of the inflammatory process. It is notable that we could not distinguish the two groups by duration or clinical activity of disease and saw only a few tissues that appeared to have areas of intense T lymphocyte infiltration superimposed on a background of fibrin-laden group B type synovial abnormalities. Alternatively, we suggest that factors in addition to the duration of the disorder may influence the synovial immunopathologic findings. These could include genetically determined factors in host response but could also include different amounts, types, and/or sources of causative stimuli. These factors remain undefined.

Our findings support the view that patient-to-patient heterogeneity is the hallmark of active rheumatoid arthritis, even among patients who are clinically indistinguishable by widely applied criteria. More important, we have demonstrated a relationship, heretofore unreported, between peripheral blood mononuclear cell unresponsiveness to soluble antigens and high intensity lymphocyte infiltration in the synovium. This apparently paradoxical relationship is consistent with other data. For example, we have also shown that anergic rheumatoid arthritis patients have depressed frequencies and absolute numbers of Leu $3A^+$ cells, increased frequencies and absolute numbers of Leu $2A^+$ cells (and thus depressed Leu 3A/Leu 2A ratios), and elevated frequencies of HLA-DR-bearing T lymphocytes in the peripheral blood. These patients respond more favorably, at least transiently, to removal of mononuclear cells by short-term repeated leukapheresis than do nonanergic rheumatoid arthritis patients (9, 51). These findings indicate that T cell subset ratios in peripheral blood and synovial tissue are inversely related; i.e., anergic patients have elevated tissue Leu 3A/Leu 2A ratios and depressed peripheral blood mononuclear cell Leu 3A/Leu 2A ratios, and nonanergic patients have the inverse. These results suggest that anergy and traffic of activated mononuclear cells between these two compartments are interrelated phenomena. This concept provides insight into potential mechanisms underlying the association of anergy and clinical improvement after repeated short-term leukapheresis (9). For example, leukapheresis may interrupt the migration of HLA-DR^+, Leu $3A^+$ cells to sites of active inflammation and thus ameliorate the inflammatory process. The observation that the beneficial effects of leukapheresis are transient supports this concept. Our data further imply that patients with active rheumatoid arthritis comprise an immunopathogenetic spectrum with polar subgroups, like that seen in certain other chronic inflammatory conditions (52–54). In addition to providing clues to the pathogenesis of rheumatoid arthritis, these studies have implications for approaches to therapy in the disorder.

Acknowledgments

The authors thank Ms. Janice Allen, Mr. William Helsel, Dr. Hsu-Ming Hsu, and Mr. Edward Soban for their invaluable advice and technical assistance.

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