AN ARTHRITOGENIC LYMPHOKINE IN THE RAT

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Although substantial evidence suggests that T cells participate in the pathogenesis of rheumatoid arthritis (1), the mechanism(s) involved is unknown. Type II collagen–induced arthritis in rats (2) and mice (3) is an inflammatory synovitis, aspects of which can be passively transferred by immunocompetent cells (4, 5) or IgG antibodies (6–8). The recognition that squirrel monkeys are susceptible to collagen arthritis (9) has created further interest in the potential relevance of this model for human disease. Rats with collagen arthritis develop antigen-specific cellular sensitivity to type II collagen, as measured by in vitro (10–12) and in vivo (13, 14) assays. This paper reports the identification and partial physicochemical characterization of a type II collagen–induced lymphokine that is arthritogenic in rats.

Materials and Methods

Animals. 100–125-g rats from the following inbred strains were used in separate experiments: female Wistar-Furth (W-F) (Microbiological Associates, Walkersville, MD); female Louvain-MN (LOU-M) and LOU-C of either sex (Small Animal Facility, National Institutes of Health, Bethesda, MD); and female Listar (LIS) from a colony maintained at the Animal Resource Center of Harvard Medical School (Boston, MA). Histocompatibility in all strains was verified using mixed lymphocyte culture techniques (4, 15). Male DBA/2 mice weighing 25–30 g were purchased from the Jackson Laboratory (Bar Harbor, ME).

Collagen. Type II collagen, prepared by pepsin treatment of lathyritic chick sternal cartilage by a method previously described (2, 13), was either obtained from a commercial source (Genzyme Corp., Boston, MA) or purified in our laboratory. Type I collagen had been previously prepared (16), using pepsin, from the skin of lathyritic chicks. Collagens were tested for purity by amino acid analysis and disc sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)[1] (17). Except for the portions of type II collagen that were thermally denatured (2), preparations were in the native state.

Sensitization. Sensitization was achieved by injecting rats intradermally with 0.4 mg of

[1] *Abbreviations used in this paper:* AF, arthritogenic factor; BS, Blue-Sepharose; Con A, concanavalin A; DFP, diisopropylphosphofluoridate; IL, interleukin; LIF, leukocyte inhibitory factor; LNC, lymph node cells; OD, optical density; PG, prostaglandin; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

collagen solubilized in 0.5 ml 0.1 M acetic acid, and emulsified in 0.5 ml incomplete Freund's adjuvant (2), or 0.25 mg ovalbumin (grade V; Sigma Chemical Co., St. Louis, MO) solubilized in 0.5 ml 0.9% NaCl and emulsified in 0.5 ml complete Freund's adjuvant (12). To stimulate lymph node hypertrophy for experiments requiring nonsensitized cells, rats were injected intradermally with 0.5 ml of 0.9% NaCl emulsified in 0.5 ml of incomplete Freund's adjuvant (12).

Cell Preparation. Lymph node cells (LNC) were obtained at sacrifice 14–21 d after antigen injection, and subjected to Ficoll-Hypaque centrifugation to facilitate removal of nonviable cells (12). Nylon wool–passaged LNC and plastic-adherent LNC (17) were used as sources of T cell–enriched and monocyte/macrophage–enriched populations, respectively. <1.5% of the nylon wool–eluted population showed positive staining using a fluorescein-conjugated goat anti–rat gamma globulin (E.Y. Pharmaceuticals, San Mateo, CA) (18), whereas >95% of the plastic-adherent cells were positive by esterase staining (17). Type II collagen–coupled spleen cells, which are capable of inducing antigen-specific delayed hypersensitivity when injected subcutaneously, were prepared using ethylcarbo-diimide (16).

Cultures. LNC were cultured in Corning 25200 tubes (Corning Glass Works, Corning, NY) at a density of 10^7 cells/ml in medium 199 (Microbiological Associates), supplemented with penicillin-streptomycin (100 U and 100 μg/ml, respectively), buffered with 20 mM Hepes solution (Gibco Laboratories, Grand Island, NY), and made to contain 2% heat-inactivated fetal calf serum (Lot 3656; IBL Tissue Culture Products, Rockville, MD), for 48 h at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 in the presence or absence of concentrations of concanavalin A (Con A), phytohemagglutinin-M (PHA), or antigen found to be optimal for the generation of rat leukocyte inhibitory factor (LIF) (12). T cell– and monocyte-enriched populations were cultured under these conditions at densities of 10^7 and 10^5 cells/ml, respectively. Cell viability (12) exceeded 95% at culture onset. After 48 h, supernatants were pooled, clarified by centrifugation (12,000 g for 20 min), dialyzed at 4°C against 0.9% NaCl and water for an additional 48 h, and concentrated to a volume of ~1.0 ml per 10^9 cells by vacuum dialysis or lyophilization. Vacuum-dialyzed material was tested immediately, and lyophilized material was stored at −20°C (12).

Establishment of Antigen-reactive T Cell Lines. T cell lines were established using the method of Ben-Nun et al. (19). LOU-M or -C LNC were obtained 7–10 d postimmuni-zation with type II collagen or ovalbumin and suspended at a density of 5×10^6 cells/ml in Dulbecco's modified Eagle's medium (Microbiological Associates), supplemented with 1% fresh rat serum, 2-mercaptoethanol (5×10^{-5} M), penicillin-streptomycin, and Hepes. LNC were cultured in 100-mm petri dishes (6 ml/dish) with antigen (12). After 72 h of incubation, the cells were harvested and the lymphoblasts separated by centrifugation using a discontinuous Ficoll gradient (19). A fraction containing >90% lymphoblasts was found consistently in the interface between densities of 1.06 and 1.05 g/ml. These cells were recovered by pipetting, washed twice, and cultured at a density of 2×10^5 cells/ml in Dulbecco's modified Eagle's medium supplemented with 20% (vol/vol) of supernatant containing interleukin 2 (IL-2), 10% horse serum (Gibco Laboratories), 2-mercaptoetha-nol, nonessential amino acids, sodium pyruvate, antibiotics, and Hepes, without added antigen (10 ml/dish) (20). The IL-2-containing supernatant was prepared using Con A–stimulated DBA/2 splenocytes (20). The isolated lymphoblasts that were propagated represented ~1% of the starting LNC population.

After 7 d, cells undergoing further selection were harvested and resuspended in the culture medium at a density of 2×10^5 cells/ml together with 10^7 cells/ml irradiated (1,500 rad) syngeneic accessory cells (from the spleen of naive rats) and antigen (collagen or ovalbumin). After 72 h of incubation, the cells were collected, washed, and applied to a Ficoll-Hypaque gradient to isolate the viable lymphoblasts (12). The lymphoblasts were resuspended in propagation culture medium (2×10^5 cells/ml) and reseeded (10 ml/dish). After 3–4 d of incubation, the cells were harvested and restimulated with antigen as described above. Supernatants used in this study were collected on either day 20 or 56, which was at the end of an antigen-stimulation cycle. T cell membrane markers were

identified as described (18), using the murine monoclonals, W3/13, W3/25, and OX8 (Accurate Chemical and Scientific Corp., Westbury, NY) and fluorescein-conjugated F(ab')$_2$ fragments of goat anti–mouse IgG (Fc fragment–specific) (Cappel Laboratories, Cochranville, PA).

Thymidine Incorporation Assay. Proliferation responses of line cells to Con A and antigen stimulation were measured by a thymidine incorporation technique adapted from one used previously (10, 13). Line cells were incubated in round-bottomed microtiter plates in quadruplicate wells. Each well contained 2.5×10^4 cells plus 10^6 accessory cells consisting of irradiated (1,500 rad) syngeneic spleen cells in 0.2 ml Dulbecco's modified Eagle's medium with 1% fresh rat serum in the presence or absence of antigen/mitogen at described concentrations (12, 13). After 48 h of incubation, cells in each well were pulsed with 1 μCi [^{3}H]thymidine (sp act, 5.0 Ci/mM; Amersham Corp., Arlington Heights, IL), and incorporation was measured 18 h later (21). Results are expressed as a stimulation index, calculated as (mean cpm with antigen or Con A)/(mean cpm without antigen or mitogen).

Gel Filtration. Lyophilized supernatant material was fractionated on a calibrated column (2.5×100 cm) containing Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) and was subsequently processed as described (12).

Affinity Chromatography. Supernatant material, derived from serum-containing cultures of nylon wool–purified cells or line cells, was depleted of albumin using the method of Travis et al. (22). 40 mg of lyophilized crude supernatant material was solubilized in 0.05 M Tris-HCl with 0.1 M KCl, pH 7.0, and applied to a preequilibrated 0.9×30 cm column containing Blue–Sepharose CL-6B (BS) (Pharmacia Fine Chemicals). Fractions of unbound material with an optical density at 280 nm (OD$_{280}$) of >0.05 were pooled (BS peak I). Bound material, including albumin, was eluted with 0.05 M Tris-HCl and 0.2 M NaSCN, pH 8.0 (BS peak II). Both pools were vacuum dialyzed to about one twentieth their original volume. Depletion efficiency was assessed by radial immunodiffusion, using 1.5% agarose containing a 1:50 dilution of rabbit anti–horse albumin (Cappel Laboratories) or rabbit anti–bovine albumin (Cappel Laboratories), where appropriate. Up to 96% of the albumin was removed from BS peak I by this procedure.

For the final affinity isolation, collagen was linked to CNBr-activated Sepharose 4 B (Pharmacia Fine Chemicals), using the method of Stuart et al. (6). Once the ligand (type I or II collagen) had been bound and the gel reequilibrated with the coupling buffer (0.01 M phosphate-buffered saline with 0.5 M NaCl), 4 ml of BS peak I in phosphate-buffered saline was added to the gel and incubated overnight at 4°C with rocking. A 1×6 cm column was filled with the gel, washed with phosphate-buffered saline until the OD$_{280}$ was <0.01, and fractions were pooled (CNBr peak I). A 0.2 M glycine-HCl wash, pH 2.8, was pumped onto the column and allowed to remain for 3 h before eluting. These latter fractions were also pooled (CNBr peak II). Elution profiles were monitored at OD$_{280}$ and concentrations determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA), with bovine serum albumin as a standard (23). Both pools were dialyzed for 48 h against distilled water, lyophilized, and stored at −20°C.

SDS-PAGE. Discontinuous SDS-PAGE was performed as described by Laemmli (24). All samples were run under reduced conditions using 2-mercaptoethanol. 10% polyacrylamide, 1.5-mm-thick slab gels were stained by a silver nitrate method, which detects quantities of protein of ≥10 ng (25). Relative molecular weight (M_r) was determined using the following Bio-Rad protein standards: phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (50,000 and 45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

Immunoglobulin Preparation. Material enriched for Ig was prepared by ammonium sulfate precipitation (16) of pooled serum, obtained at sacrifice from the rats that developed collagen arthritis, or from immunologically naive rats, and stored at −20°C. The IgG antibody titer to native type II collagen was measured by an enzyme-linked immunosorbent assay (26).

Intraarticular Injections. Crude or G-100-fractionated supernatant and Ig-enriched material were resuspended in Hanks' balanced salt solution (Microbiological Associates)

at a ratio of 1:1 (vol/vol). Protein concentrations of the crude supernatants were ~700 mg-percent, as measured by the Lowry technique (27). Affinity-purified supernatant material was resuspended in Hanks' balanced salt solution at a concentration of ≤10 mg-percent protein. Suspensions were membrane (0.45 μm)-sterilized. Under aseptic conditions, 200 μl of the crude or G-100 suspension was injected through the avascular region of the infrapatellar ligament (28) into one knee of a syngeneic naive rat, lightly anesthetized with ether, using a 26-gauge needle. For affinity-purified supernatants, 100 μl was injected.

Arthritis Assessments. After knee injection, rats were evaluated at least every other day for overt clinical signs of arthritis, i.e., a limping gait or obvious knee-joint swelling. Unless specified, rats were sacrificed 7 d postinjection. Synovial tissue overlying the infrapatellar fat pad and the contiguous margins of the patellar and/or tibial cartilage were removed and blocked in paraffin; multiple 5–7-μm-thick sections were obtained and either stained with hematoxylin and eosin, or processed for immunofluorescence (28). Based upon morphologic studies in collagen arthritis (2, 4, 28, 29), and a method used by Andreis et al. (30) in a rabbit-synovitis model, a scoring system was devised for the hematoxylin and eosin–stained slides to reflect (*a*) the magnitude of diffuse synovial membrane thickening (<5 cell layers thick = 0; 5–10 cell layers = 1; 10–15 cell layers = 2; >15 cell layers = 3), and (*b*) the degree of inflammatory cell (mononuclear cells and polymorphonuclear leukocytes) infiltration and neovascularization in the subsynovial layers (absent = 0; minimal = 1; moderate = 2; marked = 3). Each of the two categories was assigned an integer, resulting in a maximum histologic score of six. Slides were read by the same observer, who was unaware of the experimental group from which the tissue had been obtained; at least five sections were evaluated from each knee.

Physicochemical Treatments. G-100-fractionated supernatant material was exposed to heat, chymotrypsin (a proteinase with broad specificities), and to the serine esterase inhibitor, diisopropylphosphofluoridate (DFP) under conditions previously used to characterize rat LIF (12). In other experiments, LNC from arthritic rats were cultured in the presence or absence of the endotoxin inactivator, polymyxin B (5 μg/ml) (17), or puromycin, which was used at a nonlethal concentration to block de novo protein synthesis, and removed by methods already described (12).

LIF Assay. LIF activity in G-100-fractionated material was measured by its ability to inhibit the migration of rat polymorphonuclear leukocytes from capillary tubes. Activity generated by Con A provided a control (12).

Complement Depletion. Cobra venom factor was prepared and analyzed by the method of Salant et al. (31). Rats were injected intraperitoneally with 75 and 25 U (32) cobra venom factor on day -1 and day 4, respectively, and intraarticularly with G-100-fractionated material on day 0. Serum C3, measured by Mancini single radial immunodiffusion (33), was undetectable in all of the rats on day 4, and was <45% of the pretreatment value on day 7, when the rats were sacrificed.

Statistical Analyses. Continuous variables were analyzed by their group means (Student's *t*-test) and dichotomous variables by their proportionate group frequencies (x^2 test).

Results

Identification of Arthritogenic Factor (AF). As shown in Table I, a significant synovitis developed in the injected knees of W-F rats receiving the crude supernatant material from cultures of LNC or T cells from collagen-arthritic rats stimulated in vitro with native type II collagen. Although ovalbumin is a potent cellular immunogen in rats (12, 16), injection of supernatants derived from ovalbumin-sensitized LNC did not create this lesion. Only minimal inflammation developed following injection of Ig containing antibodies to type II collagen. The inability of collagen-coupled cells, as well as supernatants derived by culturing LNC from nonarthritic cells or macrophage-enriched cell populations with native type II collagen, to induce appreciable synovitis suggests that collagen carryover does not explain the results of these experiments.

TABLE I

Histologic Assessment of Synovial and Subsynovial Tissue in Knees from W-F Rats Injected with Crude Supernatant or Serum-derived Material

Antigen	Donor*	Culture		Material injected	Histologic score $(n)^{\ddagger}$	P
		Cell	Stimulus			
Type II	Arthritic	LNC	Type II	Supernatant	$3.9 \pm 0.5^{\S} (14)^{\parallel}$	—
Type II	Arthritic	LNC	Denatured type II	Supernatant	2.0 ± 0.6 (8)	$0.03^{\P}$
Type II	Nonarthritic	LNC	Type II	Supernatant	1.6 ± 0.3 (10)	$0.002^{\P}$
Type I	Nonarthritic	LNC	Type I	Supernatant	1.7 ± 0.3 (10)	$0.003^{\P}$
Type II	Arthritic	LNC	Con A	Supernatant	0.7 ± 0.7 (7)	$0.0001^{\P}$
Type II	Arthritic	LNC	PHA	Supernatant	1.5 ± 0.5 (4)	$0.03^{\P}$
Type II	Arthritic	T cell	Type II	Supernatant**	3.5 ± 0.6 (6)‡‡	—
Ovalbumin	Nonarthritic	T cell	Ovalbumin	Supernatant**	1.5 ± 0.3 (4)	$0.05^{\S\S}$
Type II	Arthritic	Macrophage	Type II	Supernatant$^{\parallel\parallel}$	1.2 ± 0.5 (4)	$0.02^{\S\S}$
—	—	—	—	Type II–coupled cells¶¶	1.5 ± 0.5 (4)	$0.03^{\S\S}$
—	—	—	—	Arthritic serum fraction***	2.1 ± 0.4 (8)	$0.03^{\P}$
—	—	—	—	Nonarthritic serum fraction‡‡‡	2.0 ± 0.3 (6)	$0.03^{\P}$
—	—	—	—	Nonimmune serum fraction	1.2 ± 0.6 (5)	$0.01^{\P}$
—	—	—	—	Hanks' balanced salt solution	0.4 ± 0.4 (5)	$0.001^{\P}$

* 70% of the rats developed arthritis 10–16 d after immunization with native type II collagen. In these and previous experiments (2, 10), no arthritis resulted when rats were immunized with the other antigens.

$\ddagger$ n represents the number of rats in which the injected knee was evaluated.

$\S$ Histologic scores are given as mean $\pm$ SEM for each group.

$\parallel$ One rat developed limping and swelling of the knee.

$\P$ Significance compared to the score produced by the supernatant from the arthritic LNC cultured with type II collagen.

** Knees received material derived from 10^8 nylon wool–purified cells.

$\ddagger\ddagger$ Two rats developed limping and swelling of the knee. Except for those mentioned, none of the other injected knees in these experiments exhibited clinical signs of arthritis.

$\S\S$ Significance compared to the score produced by the supernatant from the arthritic T cells cultured with type II collagen.

$\parallel\parallel$ Knees received material derived from 2.5×10^5 plastic-adherent cells.

$\P\P$ 5×10^7 cells in Hanks' balanced salt solution injected.

*** IgG antibody titer, 12 serial twofold dilutions.

$\ddagger\ddagger\ddagger$ Antibody titer, eight serial twofold dilutions.

To estimate the molecular mass of the lymphokine responsible for this inflammatory activity, lyophilized material from arthritic W-F LNC cultured with type II collagen underwent Sephadex G-100 filtration. The eluate from 10 columns was divided into three fractions (high molecular mass fraction, 90–60 kilodaltons [kD]; intermediate fraction, 60–23 kD; and low molecular mass fraction, <23 kD). 9 (41%) of 22 rats injected with the high fraction, vs. 1 of 15 rats injected with the intermediate fraction, and none of 18 rats injected with the low molecular mass fraction developed overt clinical signs of knee arthritis ($P < 0.01$ for both comparisons) (Fig. 1). The mean histologic score of rats injected with the high-mass fraction was significantly greater than the scores of the other two groups (Table II). Additional subfractionation of G-100-derived material indicated that AF activity eluted in the 60–70 kD range. Other studies depicted in Table II showed that AF was an in vitro–synthesized protein, distinct from the similarly sized lymphokine, LIF (12), and not inactivated at 56°C; its arthritogenicity was not likely to be due to contamination by endotoxin. Complement depletion with cobra venom factor did not affect the response of rats to AF.

Affinity Purification of AF. The inability of mitogens and antigens other than native type II collagen to generate arthritogenic supernatants (Table I) suggested that AF is an antigen-specific lymphokine. Accordingly, W3/25+ T cell lines were established from collagen-immunized LOU-M and LOU-C rats that reacted

FIGURE 1. Swollen knee (viewer's right) 7 d after injection of G-100-enriched AF, compared to the uninjected knee, of a sacrificed rat in which the overlying skin has been reflected.

specifically to type II collagen (Table III). The LOU strain was selected because of its high degree of susceptibility to collagen arthritis observed in other (unpublished) studies. An additional W3/25$^+$ line was established from LOU-M rats immunized with ovalbumin and CFA by stimulation with ovalbumin; this line reacted to antigenic challenge with both ovalbumin and mycobacteria.

The supernatant materials derived from these cell lines were used in the following affinity chromatography studies. The two collagen-reactive T cell lines produced material that bound to type II collagen, and when eluted, gave pronounced AF responses in the knee assay (Table IV). This antigen-binding AF activity was also generated by nylon wool–nonadherent cells obtained from arthritic W-F and LIS rats. No binding occurred when this material was passaged through an affinity column prepared with type I collagen. The ovalbumin/mycobacteria–reactive line did not produce collagen-binding AF activity. Material enriched for lymphokines by stimulating naive nylon wool–nonadherent cells with Con A and subjecting the supernatant to attempted affinity purification on the type II collagen column was also devoid of AF activity. This result, as well as the outcome of the ovalbumin/mycobacteria line experiment, make it unlikely that collagen leached off the affinity column produced the inflammation in these studies.

SDS-PAGE under reducing conditions showed a single protein band in the T cell–derived AF containing materials eluting from the type II collagen–Sepharose column (Fig. 2). Similar to the molecular mass estimates by gel filtration, the

TABLE II

Studies of Rat AF in W-F Rats

Material tested	Histologic score (n)	P
Fractionated AF (kD)		
60–90	3.8 ± 0.5 (22)*	—
23–60	1.8 ± 0.4 (15)‡	0.01
<23	1.1 ± 0.2 (18)	0.001
60–70	2.8 ± 0.3 (11)‡	—
50–60	2.0 ± 0.4 (8)	NS§
70–80	1.0 ± 0.3 (11)	0.0001
80–90	0.7 ± 0.3 (11)	0.001
Normal recipient	2.9 ± 0.5 (7)	—
Complement-depleted recipient	2.8 ± 0.8 (4)	NS
Incubation without chymotrypsin	2.9 ± 0.5 (7)	—
Incubation with chymotrypsin	0.7 ± 0.3 (3)	0.02
Incubation without DFP	2.0 ± 1.0 (2)	—
Incubation with DFP‖	2.0 ± 0.0 (2)	NS
No heat	2.2 ± 0.5 (4)	—
Heat (56°C, 30 min)	2.0 ± 0.6 (5)	NS
Heat (80°C, 30 min)	0.6 ± 0.6 (5)	NS
Unfractionated material¶		
No drug	3.9 ± 0.5 (14)‡	—
Polymyxin B	3.5 ± 0.6 (4)	NS
Puromycin	1.7 ± 0.5 (4)	0.05

Designations are identical to those described in Table I.

* Nine rats developed limping and swelling of the knee.

‡ One rat developed limping and swelling of the knee. Except for those mentioned, none of the other injected knees in these experiments exhibited clinical signs of arthritis.

§ NS, not significant.

‖ The resistance of AF to DFP indicates that, unlike rat LIF, this protein is not a serine esterase (12). In addition, this fractionated AF preparation did not possess LIF activity (mean migration inhibition was 8% vs. 54% for AF and Con A–induced LIF, respectively).

¶ Obtained by culturing arthritic LNC with type II collagen in the presence or absence of a drug.

affinity-purified AF from the T cell lines and the nylon wool–purified T cell populations possessed an M_r of 65,000 by SDS-PAGE.

Characteristics of Lesion Induced by AF. Pannus formation, neovascularization, and synovial infiltrates, consisting predominantly of mononuclear inflammatory cells, some polymorphonuclear leukocytes, and a few eosinophils, were found as early as 48 h after injection of G-100-enriched AF into the knees of W-F rats. Cartilage erosion was also noted at this time (Fig. 3). Granulomatous areas were occasionally observed in the pannus of day 7 lesions induced with G-100-enriched AF, and immunofluorescence evaluation of synovia during the period 2–7 d

TABLE III

Thymidine Incorporation Responses by T Cell Lines

Strain	Propagating antigen	Background (cpm)	Stimulation index with antigen or mitogen					
			Type II collagen	Type I collagen	Oval-bumin	PPD*	Myco-bacte-ria	Con A
LOU-M	Type II	398 ± 97	16.8	0.7	0.8	0.4	ND	7.8
LOU-C	Type II	199 ± 16	1.5	ND	ND	0.9	ND	1.3
LOU-M	Ovalbumin	15,772 ± 830‡	1.1	ND	2.4	1.2	2.0	3.6

Measured in quadruplicate by 3-d cultures of aliquots of 2.5×10^4 line cells and 10^6 syngeneic accessory cells, obtained from the spleen and irradiated with 1,500 rad. The cells were cultured in the presence of 10 μg/ml of antigen or mitogen and 1% fresh autologous serum. Line cells were tested for [^{3}H]thymidine incorporation 17 d after onset of primary culture, and showed 95–98% staining positivity for the W3/25 antigen at this time. ND, not done.
* PPD, purified protein derivative of tuberculin.
‡ This high degree of proliferation in the resting state is similar to that observed (47, 48), on occasion, in other established rat T cell lines.

TABLE IV

Assessment of Knees Injected with Cell-derived Material Tested for Ability to Bind Type II Collagen

Antigen	Donor	Strain	Culture		Affinity column	Histologic score‡ (n)	P
			Cell*	Stimulus			
Type II	Arthritic	W-F	T	Type II	Type II	4.1 ± 0.3 (5)	0.001
Type II	Arthritic	LIS	T	Type II	Type II	4.1 ± 0.3 (4)	0.003
Type II	Prearthritic	LOU-M	T line	Type II	Type II	4.3 ± 0.4 (6)	0.001
Type II	Prearthritic	LOU-C	T line	Type II	Type II	3.6 ± 0.2 (6)	0.001
Type II	Arthritic	W-F	T	Type II	Type I	1.6 ± 0.5 (5)	NS
Ovalbumin	Nonarthritic	LOU-M	T line	Ovalbumin	Type II	1.4 ± 0.4 (6)	NS
—	Naive	W-F	T	Con A	Type II	1.0 ± 0.6 (6)	—

Designations are identical to those described in Tables I and II.
* Cell culture represents either nylon wool–purified T cells or an aliquot of cells from a T cell line obtained 17 (LOU-M) or 53 d (LOU-C) after the onset of culture.
‡ Derived from tissue samples 7 d after injection of 100 μl of glycine-HCl eluate from the native collagen–Sepharose column. Protein concentrations in the eluates were ≤ 0.1 mg/ml.

postinjection detected abundant fibrin, and rare deposits of Ig and complement (C3). Histologically, the synovitis induced in the knees of LOU rats by the affinity-purified AF was similar to that observed using G-100-enriched material (Fig. 4). Four knees of LOU-M rats injected 25 d earlier with affinity-purified, line cell–derived AF showed hyperplasia of the synovial lining, mononuclear cell infiltration, subsynovial fibrosis, and occasional granulomas (mean histologic score ± SE, 3.6 ± 0.3).

Discussion

These data demonstrate the existence of AF, a novel T cell–derived arthritogenic lymphokine, in different strains of rats. At least a portion of this arthritogenic activity is antigen binding, and the ability of native type II collagen to affinity purify AF activity suggests that the responsible species is an antigen-
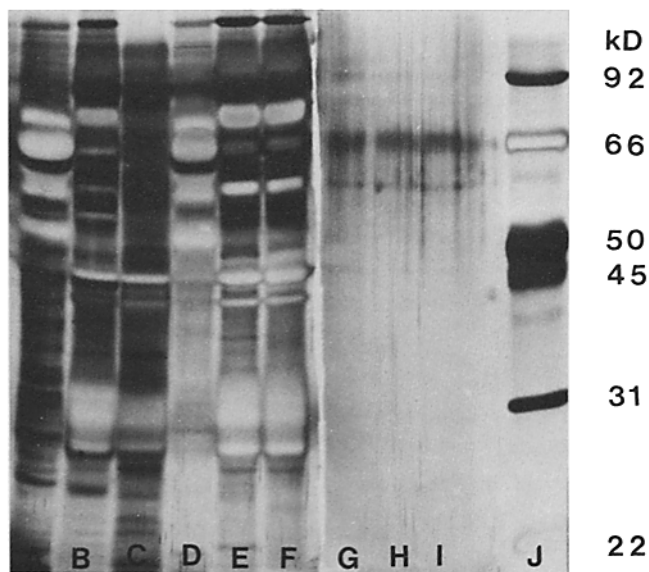
FIGURE 2. Analysis of AF by SDS-PAGE and silver staining under reducing conditions. Lanes represent BS-peak I. *A*, AF-containing material derived from culturing nylon wool–enriched T cells from arthritic LIS rats with type II collagen, diluted for application to the type II collagen–Sepharose column; *B*, AF-containing material generated by the type II collagen–specific LOU-M T cell line, diluted for column application; *C*, diluted AF-containing material from the collagen-reactive LOU-C T cell line; *D–F*, CNBr peak I from the LIS, LOU-M, and LOU-C material, respectively; *G–I*, CNBr peak II (glycine-HCl eluate) from the LIS, LOU-M, and LOU-C material, respectively; *J*, molecular mass standards. The linear streaks across the gel, including the one in the 60 kD region, are artifacts seen in similar gels run without protein application. 6–12 μg of protein was applied to lanes *A–F*, whereas lanes *G–I* each received <1 μg. Development of this gel was prolonged to further demonstrate the absence of contaminating bands in lanes *G–I*. The analyzed CNBr peak II material from the W-F T cells also had a molecular mass of 65 kD.

specific lymphokine reactive with epitopes on type II collagen. The ability of puromycin to block the generation of AF activity in vitro indicates that the protein is not preformed. Further analytic and functional characterization of AF is required to ascertain with greater certainty whether or not AF exists solely as a single 65 kD polypeptide in the rat. Additional work is also necessary to determine whether the bioactivity of AF crosses strains and/or species, or is major histocompatibility complex–restricted.

Most studies of antigen-specific T cell–derived factors have identified those with suppressor properties (34, 35), however, analogous factors with promoting activities have been detected in several murine systems (36–38). The ability of native, but not denatured, type II collagen to stimulate degrees of AF activity that are readily detectable by bioassay suggests that rat T cells capable of recognizing quarternary epitopes on collagen exist. Previous studies in collagen arthritis have provided additional indirect evidence supporting this proposal (10, 16). Murine T cells reactive with conformationally dependent determinants on insulin have been identified recently by Glimcher et al. (39), providing precedence for such a phenomenon.

Although the collagen-reactive LOU-M and LOU-C T cell lines differed

FIGURE 3.   Erosion of cartilage (arrow) seen 48 h after injection of G-100-enriched AF. (Original magnification, × 200).

markedly in their ability to respond in vitro to native type II collagen by thymidine incorporation, both lines generated statistically similar degrees of AF activity. This finding provides evidence that the capacity to proliferate and produce lymphokines in response to antigen stimulation by various T cell lines is dissociable, although the basis for this phenomenon is unclear.

The molecular mass of AF differs from the ~20 kD protein, catabolin (40), which stimulates chondrocytes in vitro, nor is AF identical to the ~15 kD collagen-inducible cytokine, IL-1 or mononuclear cell factor, which heightens release of collagenase and prostaglandin E_2 (PGE_2) by cultured human synovial cells (17) and mediates cartilage matrix degradation (41). A substance, functionally equivalent to mononuclear cell factor in the synoviocyte assay, can be generated in rats by stimulating naive LNC with Con A, but its M_r by G-100 filtration is 10,000–20,000 (C. E. Brinckerhoff and D. E. Trentham, unpublished data). Another difference from mononuclear cell factor is the inability of G-100 fractionated AF to stimulate release of collagenase and PGE_2 in the in vitro rat synoviocyte system (C. E. Brinckerhoff, S. M. Helfgott, and D. E. Trentham, unpublished data).

In rats, the erosive joint lesion that results from intravenous injection of affinity-purified collagen antibodies has been reported (8) to be complement-dependent, and to consist largely of polymorphonuclear leukocyte infiltration, rather than pannus formation. The apparent lack of involvement of blood complement and the mononuclear inflammatory cell preponderance in the AF-
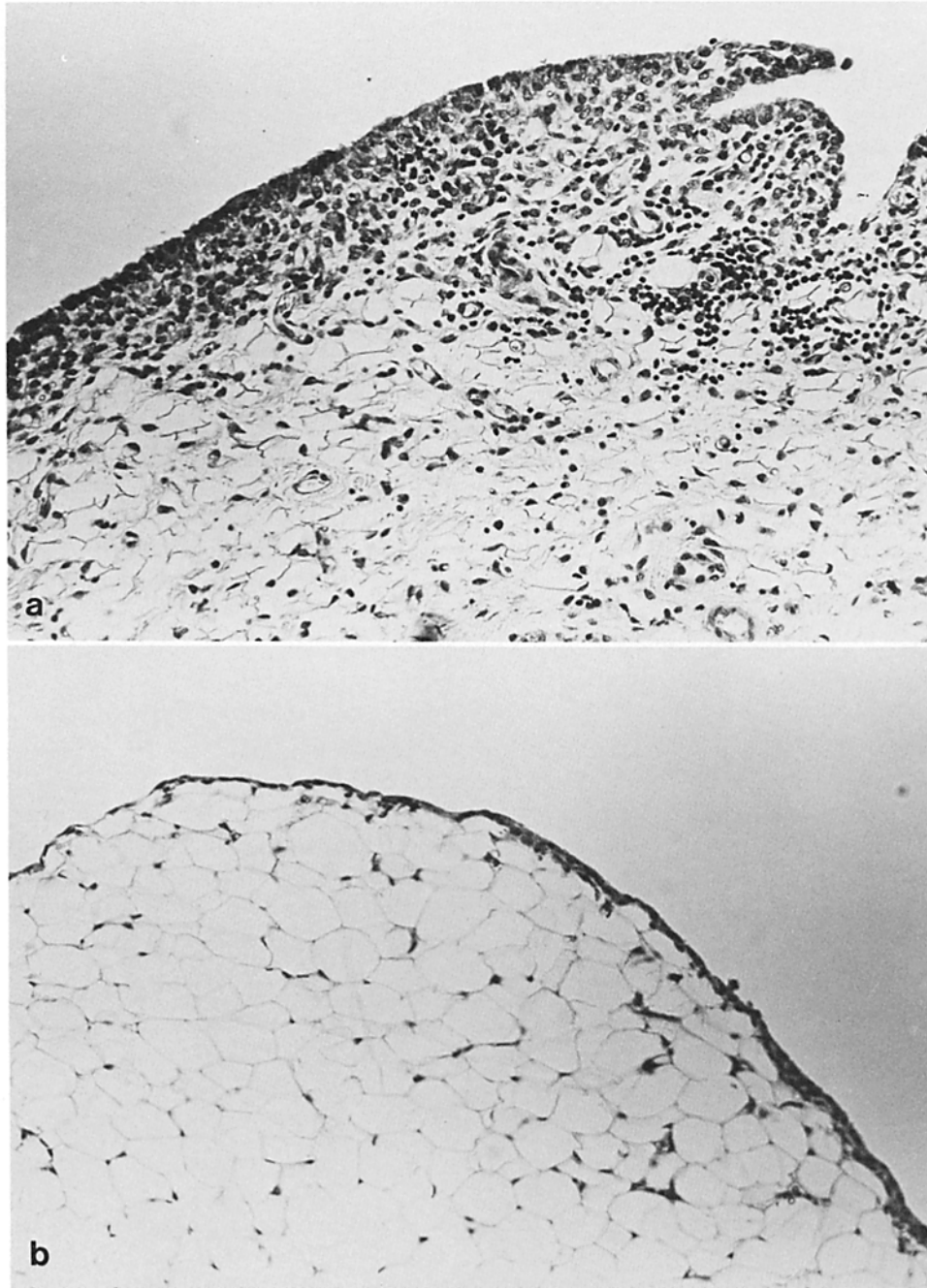
FIGURE 4. (a) A hypertrophied and inflamed synovium induced by injecting (7 d earlier) ~10 µg of affinity-purified AF, generated by the type II collagen–specific LOU-M T cell line, vs. (b) a normal rat knee synovium. (Original magnification, × 400).

induced lesion make it unlikely that antibody production by the recipient accounts for the arthritogenic effect of AF.

Although the initiation of collagen arthritis has been postulated (6, 7) to be an antibody-mediated event, the lesion produced by antibody is transient (6–8), and is incapable of being sustained by repeated injections (42). In contrast, a persistent arthritis characterized by extensive synovial hypertrophy can be induced by the intravenous injection of collagen-sensitized spleen cells and LNC in rats (4), or by injection of nylon wool–purified T cells in mice (5). In the recipients of cells, no serum antibody response to collagen was detected (4, 5). These passive transfer experiments may indicate that cellular immunity plays a major role in the pathogenesis of collagen arthritis. The recognition of AF identifies a mechanism whereby this process could be subserved.

Increasing attention is being directed to the role of T cells in the pathogenesis of rheumatoid arthritis (1). Lymphokine-like moieties have been identified in the synovial fluid of patients with this disease (43). An inflammatory synovitis of the knee can be induced in rabbits by repeated intraarticular injections of culture supernatants containing factors generated by homologous LNC sensitized to keyhole limpet hemocyanin (30). The identification of AF in an animal model, and the presence of cellular sensitivity to collagen in patients with rheumatoid arthritis (44–46) are consistent with the hypothesis that this lymphokine is an effector molecule in human synovial disease.

Summary

A type II collagen–specific arthritogenic lymphokine has been identified in the rat. Arthritogenic factor (AF) is a 65 kD protein generated in vitro by T cells from rats with collagen arthritis, and it induces an erosive, proliferative synovitis when injected into the knee joint of syngeneic naive recipients. Complement does not appear to be required. These data identify a potential T cell–mediated effector mechanism in this model, and suggest that AF may function in other inflammatory synovial diseases.

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