# STREPTOCOCCAL CELL WALLS AND
# SYNOVIAL CELL ACTIVATION

## Stimulation of Synovial Fibroblast Plasminogen
## Activator Activity by Monocytes Treated with Group A
## Streptococcal Cell Wall Sonicates and Muramyl Dipeptide*

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In spite of decades of investigation, the etiology(ies) of rheumatoid arthritis (RA)[1] and many of the related rheumatic diseases remains unknown. One hypothesis suggests that certain poorly degradable bacterial peptidoglycans are transported to the joint and are capable of instigating an abnormal immune response on the part of the host (1, 2). The resultant inflammatory response by the host is self-perpetuating and leads to the destruction of the joint. This viewpoint is supported by the observation that there is an increased humoral response to bacterial mucopeptide antigens in juvenile RA patients (3, 4) and at least one report (5) of increased antibodies in adult RA patients.

Experimentally, a number of investigators (reviewed by Schwab et al. [6]) have demonstrated certain forms of chronic relapsing arthritis in animals using multiple or single injections of microbial antigens. In a rat model of arthritis (7, 8), intraperitoneally injected sonicated cell wall material from the group A streptococcus has been found to localize in the joint, initially being found extracellularly as well as inside phagocytic cells. As the arthritis became chronic, the material was found in the synovium predominantly inside the macrophages. The bacterial cell wall is composed of various polysaccharides linked to a peptidoglycan backbone, and the relative nonbiodegradability of bacterial peptidoglycan appeared to be an important patho-

[1] *Abbreviations used in this paper:* AMML, acute myelomonocytic leukemia; ATFBS, acid-treated, heat-inactivated fetal bovine serum; CM, conditioned medium; Con A, concanavalin A; CWS, cell wall sonicate; FBS, heat-inactivated fetal bovine serum; IEF, isoelectric focusing; IFN, interferon; IL-1, IL-2, interleukin 1 and 2; LPS, lipopolysaccharide; MC, mononuclear cells; MCCM, mononuclear cell conditioned medium; MDP, N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide); MEM, minimum essential medium; P, plasminogen; PA, plasminogen activator; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; SA, synovial activator; SEB, staphylococcus enterotoxin B; TdR, thymidine.

genic factor. Sonication of the cell wall material greatly enhanced its arthritogenicity. The synthetic adjuvant, N-acetylmuramyl-L-alanyl-D-isoglutamine, (muramyl dipeptide, or MDP), which forms a part of the monomer of the peptidoglycan portion of bacterial cell walls, can also be arthritogenic in rats if administered appropriately (9).

Local release of proteolytic enzymes such as plasmin and collagenase could be responsible for some of the tissue damage and vascular effects associated with RA (10, 11). Plasmin is generated after interaction of another neutral protease plasminogen activator (PA), with its substrate, plasminogen (P). The PA/plasmin system has been detected in cultures of enzyme-dissociated rheumatoid tissue (10). Plasmin can degrade basement membrane (12) and cartilage (13) and activate both a latent collagenase (10) and also certain enzyme cascades likely to be of importance in the development of inflammatory responses (14–16). It was reported that conditioned medium (CM) from normal human peipheral blood mononuclear cells (PBMC) elevated the PA activity of passaged nonrheumatoid human synovial fibroblasts (11), and more recently of the passaged rheumatoid counterparts.[2] This modulation of the synovial cell PA activity by mononuclear cells (MC) was proposed as being part of the cellular interactions contributing to the properties of the aggressive, invasive, rheumatoid "pannus" (11). The activity in the mononuclear cell CM (MCCM) is here termed synovial activator (SA) activity.

We report here the PBMC, when treated either with cell wall sonicate (CWS) material from several group A streptococcus strains or also with MDP, generate elevated levels of SA activity. The CWS are most likely acting via a monocyte-macrophage in the PBMC. Preliminary evidence indicates that SA activity is distinct from interferon-$\gamma$, interleukin 1 (IL-1), and interleukin 2 (IL-2). Thus, SA activity can be generated in vitro by structures which are both known to be arthritogenic in animals (7, 8) and to be also implicated in RA (2–5).

## Materials and Methods

*Synovial Cell Culture.* As previously described (11), synovial membrane tissue was obtained aseptically from the knees of normal patients, i.e., patients free of known rheumatoid or degenerative joint disease, needing correction of internal derangements, for example, by means of meniscectomy. Synovial tissue from the hips or knees of patients with degenerative joint disease was obtained during prosthetic joint replacements. Synovial cells from these sources have similar growth and biochemical parameters (17). The techniques used for the culture of human synovial fibroblast-like cells from explant tissue were essentially those of Castor et al. (17) and have been outlined previously (11). The passaged cells are morphologically and biochemically similar to the fibroblast-like cells studied by Castor et al. (17).

*Preparation of Streptococcal CWS.* The following group A streptococcal strains were obtained from The Rockefeller University collection: S43/100 (type 6), T12/126 (type 12), D678 (type 11), and A995 (type 57). These were grouped and typed by the Lancefield method, and the cell walls isolated by methods previously described (18). CWS were prepared by the technique of Cromartie et al. (7), and were the materials used in the current study. The CWS were resuspended by vortex mixing in phosphate-buffered saline (PBS) (0.01 M phosphate buffer (pH 7.4), 0.15 M NaCl [19]). MDP was obtained from Dr. D. Braun, Ciba-Geigy Ltd., Basel, Switzerland, and dissolved in PBS before use.

*Preparation of MCCM.* For the preparation of MCCM, PBMC from citrate phosphate dextrose-treated venous blood of normal donors or from normal blood depleted of platelets, erythrocytes, and plasma were separated on Ficoll-Hypaque gradients (Pharmacia Fine Chem-

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[2] Hamilton, J. A. Plasminogen activator activities of rheumatoid and non-rheumatoid synovial fibroblasts. Manuscript submitted for publication.

icals, Div. of Pharmacia Inc., Piscataway, NJ) (11). Varing numbers of the MC were cultured at 37°C in an atmosphere of 5% $CO_2$ and 95% air in duplicate 35-mm Diam tissue culture dishes (3001, Falcon Labware, Oxnard, CA) usually for 24–48 h, either in 1 ml α-minimum essential medium (α-MEM) alone or in the presence of various stimuli, particularly CWS preparations. MCCM were collected after centrifugation of the cells and stored at −20°C before assay of the SA activity.

*Measurement of SA Activity.*    The SA activity in MCCM preparations was measured as the ability to elevate the PA levels of target human synovial fibroblasts. For this purpose, the fibroblasts were plated at $10^4$ cells/0.2 ml in $^{125}$I-fibrin-coated wells of Costar 96-well tissue culture plates (3596; Costar, Data Packaging, Cambridge, MA), prepared as previously described (19).[2] After incubation for 24–96 h in α-MEM containing 7.5% heat-inactivated (60 min, 56°C) fetal bovine serum (FBS) and 7.5% heat-inactivated newborn bovine serum, the cultures were washed twice with α-MEM and placed in α-MEM (100 μl) containing different MCCM preparations (40 μl) and supplemented with 5% of acid-treated FBS (ATFBS) (20) or 5% P-depleted ATFBS (ATFBS-P) (21). P was removed from the FBS by passage through a Sepharose-lysine affinity column (21); this procedure has been shown to remove ≥97% of the original concentration of P (20) and allows determination of the P dependence of the fibrinolytic activity. FBS was acidified (then neutralized) for this assay to remove inhibitors of fibrinolysis (20). Aliquots (40 μl) of the supernatant medium were taken from duplicate cultures at 24 or 48 h and assayed for solubilized radioactivity as a measure of fibrinolytic activity (11, 19).

Data from individual experiments are provided. Range of variation from duplicate cultures is 10–15% of the mean value. Serial dilutions of MCCM preparations were always used to ensure that any fibrinolytic activity was on the linear range of a dose-response curve for SA activity. The fibrinolytic activities shown are the P-dependent values (i.e., PA activities) because the P-independent fibrinolytic activities never amounted to more than 10% of this value (11). The PA activities measured are not from the MCCM themselves but require the interaction between the MCCM and the synovial cells (11).

### Cell Separation

ADHERENCE AND PHAGOCYTOSIS.    PBMC were plated in 1 ml α-MEM + 10% FBS for 1.5 h at 37°C in 35-mm dishes. The nonadherent cells were removed aseptically by a pasteur pipette and resuspended (3 × $10^6$/ml) in a α-MEM + 10% FBS containing excess carbonyl iron-lymphocyte reagent (Technicon Instruments Corp., Tarrytown, NY) to deplete them of residual phagocytic cells. For this purpose, the cells were rotated gently for 30 min at 37°C, the iron carbonyl removed by a magnet, and the cells washed in medium (22). The adherent cells were washed once with α-MEM before use, and any weakly adherent cells removed by this washing were discarded. The remaining adherent cells were ≥95% monocyte-macrophages by morphology (Wright-Giemsa stain) and by the ability (≥90%) to phagocytize latex particles (1-μm Diam, The Dow Chemical Co., Indianapolis, IN) (23).

T-CELL AND B-CELL DEPLETION.    PBMC were depleted of T lymphocytes by the commonly used method of E-rosette formation with neuraminidase-treated sheep erythrocytes followed by centrifugation (24). After shock lysis of erythrocytes with ammonium chloride, T cells were also recovered by a procedure previously described (24). PBMC were depleted of B lymphocytes by the widely used method involving immunoglobulin-coated sheep erythrocytes (25). This was carried out in the laboratory of Dr. N. Chiorazzi of The Rockefeller University.

*Lysozyme Assay.*    Lysozyme in the MCCM samples was assayed (26) by measuring the initial rate of lysis of a suspension of *Micrococcus luteus* (Difco Laboratories, Detroit, MI). Human lysozyme (Dr. E. Osserman, College of Physicians and Surgeons of Columbia University, New York), diluted in α-MEM or α-MEM + 5% FBS, was used as a standard.

*Interferon (IFN) Activity.*    IFN titrations were done based on inhibition of the pathogenic effect of vesicular stomatitis virus (27). All samples were tested on human fibroblasts trisomic for chromosome 21 and on bovine kidney cells. On every 96-well plate (Falcon 3040), a laboratory standard of human IFN-α calibrated against the National Institutes of Health standard for IFN-α (G-023-901-527) was assayed. The IFN levels of test samples were not adjusted to the IFN-α reference; however, the reference varied not more than 0.5 $\log_{10}$ units in different titrations. The majority of the IFN activity produced by human leukocytes in response to staphylococcus enterotoxin B (SEB) in vitro has been classified as IFN-γ (28).

*IL-2 Assay.*    This was done by the method of Mier and Gallo (29), monitoring the ability of IL-2 to stimulate [$^3$H]thymidine ([$^3$H]TdR) incorporation into T lymphoblasts.

*Preparation and Purification of IL-1 Samples.*    Buffy coat cells were obtained after leukophoresis treatment of the peripheral blood of an acute myelomonocytic (AMML) patient (30). The CM was prepared from these cells cultured at $3 \times 10^6$/ml in 5% normal human serum for 48 h in the presence of 10 µg/ml *Escherichia coli* lipopolysaccharide (LPS) (strain 055:B5) (28). The biochemical properties of normal leukocyte IL-1 and leukemic cell IL-1 have been shown to be very similar (30).

STAGE I PURIFICATION.    IL-1 activity in the leukocyte CM can be efficiently and rapidly separated from the majority of serum proteins by the procedure of hollow-fiber diafiltration and ultrafiltration (31). Briefly, IL-1 activity is separated from the serum proteins required for its preparation by passage twice through a 50,000-mol wt cut-off hollow fiber device. This procedure removes >99% of the initial protein and results in a recovery of 80% of the ≈13,000-mol wt IL-1 activity.

STAGE II PURIFICATION.    Sucrose gradient isoelectric focusing (IEF) was performed on the sample from Stage I (31). IEF fractions in the pH range 6.8–7.2 were pooled, diluted 10-fold with PBS, and dialyzed overnight to remove the remaining sucrose and ampholine. IEF of hollow-fiber-purified IL-1 from the AMML cell CM revealed a single peak of IL-1 activity with an average isoelectric point of 7.0 (30).

STAGE III PURIFICATION.    The pooled stage III sample was further purified by semipreparative polyacrylamide gel electrophoresis in 7.5% gels as previously described (31). IL-1 was recovered from the gel slices as a single peak of activity having an electrophoretic mobility at pH 8.60 of 0.16 (tracking dye = 1.00). The recovered IL-1 activity has been estimated to be purified >16,000-fold and has been judged to be active in submicrogram amounts.

*IL-1 Assay.*    IL-1 was measured in 10-µl samples as the direct stimulation of mouse thymocytes to incorporate [$^3$H]TdR using the microassay previously published (31). All results are expressed as the average of triplicate cultures. The units of IL-1 activity have been described (31). That the activity is due to IL-1, and not IL-2, is always checked by assaying for IL-2 (see above): if no IL-2 is present, then the thymocyte assay is monitoring IL-1.

*Determination of the Properties of SA Activity.*    Pooled fractions with SA activity from a Sephadex G-100 column were dialyzed against 10 mM sodium phosphate, pH 7.4, lyophilized, and adjusted with H$_2$O to 10 times the concentration. This concentrated sample was aliquoted and stored at −20°C before use.

## Results

*Generation of SA Activity by Streptococcal Cell Wall Sonicates.*    It was recently reported (11) that concanavalin A (Con A) elicits SA activity from human PBMC in both serum-free and FBS-containing cultures. Given that streptococcal cell wall fragments can be arthritogenic in rats (8), and that streptococci could belong to a class of possible etiologic agents for RA (2–5), group A streptococcal cell wall preparations were tested as generators of SA activity from PBMC. In Fig. 1, it can be seen that a sample of type 6 (T6) CWS (1–100 µg/ml) was active in both serum-free and FBS-containing medium.

As presented in Fig. 2, CWS from three other group A streptococcal strains, namely type 12 (T12), type 11 (T11), and type 57 (T57), were also active, the data presented being for serum-free medium. In other experiments, the T6 and T12 CWS were found to have comparable activity both in terms of the effective doses and the degree of stimulation attained. None of the CWS elevated the PA activity of the synovial cells directly. As can be observed in Fig. 2, adherent cells from the PMBC were a source of the SA activity; a more detailed analysis of the cell types responsible for the SA activity is provided below. For the total PBMC and for the adherent subpopulation, all of the CWS were stimulatory over the approximate range 1–100 µg/ml.
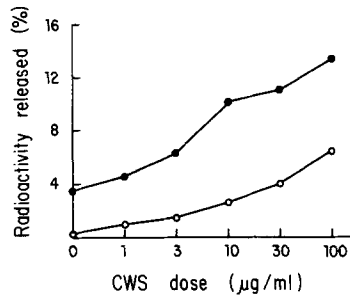
Fig. 1.   Dose response for T6 CWS generation of SA activity. Human synovial fibroblasts (2nd passage) from an osteoarthritic donor (B.S.) were plated in $^{125}$I-fibrin coated wells, and the PA activity measured after 24 h in the presence of the different MCCM preparations (Materials and Methods). MCCM were prepared from $4 \times 10^6$ PBMC in 24-h cultures. MCCM prepared in 1 ml α-MEM (O); MCCM prepared in 1 ml α-MEM + 5% FBS (●). The doses of T6 CSW shown are the final concentrations (μg/ml) in these MCCM cultures. The mean fibrinolytic activities in the wells containing α-MEM, α + 5% FBS, or 30 μg/ml T6 CWS as controls were all 0.1%.
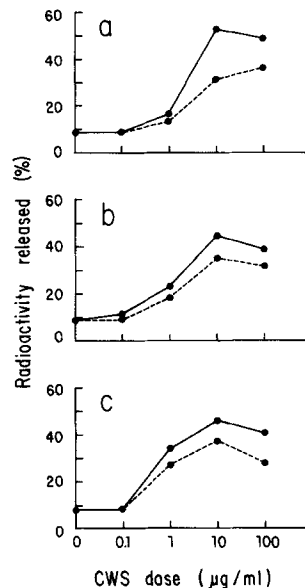


Fig. 2.   Generation of SA activity by CWS of different group A streptococcal strains. Human synovial fibroblasts (5th passage) from an osteoarthritic donor (B.S.) were plated in $^{125}$I-fibrin-coated wells, and the PA activity measured after 48 h in the presence of the different MCCM preparations (Materials and Methods). (a) T12 CWS; (b) T11 CWS; (c) T57 CWS. MCCM prepared from $4 \times 10^6$ PBMC in 48-h cultures in 1 ml α-MEM (——); MCCM prepared in the same way but from the adherent cells remaining after the 1.5-h adherence of $4 \times 10^6$ PBMC followed by washing twice with α-MEM (- - -) (Materials and Methods). The doses depicted are the final concentrations (μg/ml) of the different CWS present in these MCCM cultures. The mean fibrinolytic activities in the wells containing α-MEM or 30 μg/ml of each of the CWS materials as controls were 9%.

Other stimuli for enhanced SA activity from PBMC ($2-4 \times 10^6$ cells) in either 1 ml serum-free or in 5% FBS-containing medium include Con A (1–30 μg/ml [11], Miles-Yeda, Rehovot, Israel), phytohemagglutinin (0.1–1% vol/vol, Wellcome Reagents Ltd., Beckenham, England) and *E. coli* LPS (1 ng-10 μg/ml, strain 055:B5).

*Generation of SA Activity by MDP.* Previous reports have demonstrated that the effects of bacterial cell wall preparations can often be reproduced by molecules of N-acetylmuramic acid residues linked to small peptide structures (32, 33), which are similar to the repeating structures that go to build up the peptidoglycan structure (2). In fact, MDP has been shown to be arthritogenic in rats, provided the correct oil vehicle was included during injection (9). In Fig. 3, MDP is seen to be potent (on a weight basis) at generating SA activity from adherent cells from PBMC in the two
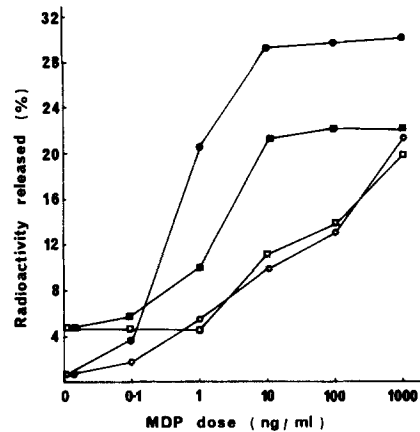


FIG. 3. Dose response for MDP generation of SA activity. Human synovial fibroblasts were plated in $^{125}$I-fibrin-coated wells. For experiment 1, the cells were from a normal donor (S.O., 3rd passage), whereas for experiment 2, the cells were from an osteoarthritic donor (B.H., 6th passage). The PA activity was measured after 48 h in the presence of the different MCCM preparations (Materials and Methods). MCCM were prepared from 48-h cultures of the adherent cells remaining after the 1.5-h adherence of 4 × 10⁶ PBMC (Fig. 2). Data from the two separate experiments are provided: experiment 1, (O, ●); experiment 2 (□, ■). Open symbols indicate MCCM prepared in 1 ml α-MEM; closed symbols indicate MCCM in 1 ml α-MEM + 5% FBS. The doses of MDP shown are the final concentrations (ng/ml) in these MCCM cultures. The mean fibrinolytic activities in the wells containing α-MEM, α-MEM + 5% FBS, or 300 ng/ml MDP as controls were 0.5% for experiment 1 and 2% for experiment 2.
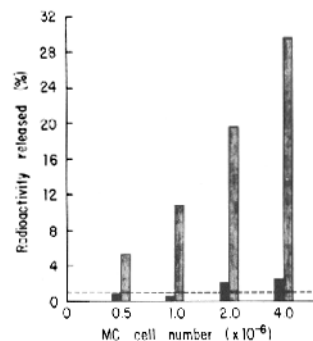


FIG. 4. Mononuclear cell number and SA activity. Human synovial fibroblasts (4th passage) from a normal donor (G.G.) were plated in $^{125}$I-fibrin-coated wells, and the PA activity measured after 24 h in the presence of the different MCCM preparations (Materials and Methods). Solid columns: MCCM prepared from different numbers of PBMC from 48 h cultures in α-MEM + 5% FBS; stippled columns: MCCM prepared in the same way but in α-MEM + 5% FBS containing 30 µg/ml T6 CWS (Materials and Methods). The mean fibrinolytic activities in the wells containing media or 10 µg/ml T6 CWS as controls were 1% (- - -).
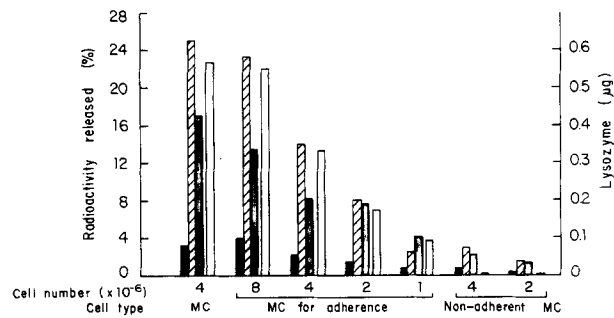
FIG. 5. Adherent cell generation of SA activity. Human synovial fibroblasts (6th passage) from an osteoarthritic donor (O.H.) were plated in $^{125}$I-fibrin-coated wells, and the PA activity measured after 24 h in the presence of the different MCCM preparations (Materials and Methods). "MC" refers to the total PBMC population and "nonadherent MC" refers to the nonadherent cells left after also removing phagocytic cells by the iron carbonyl treatment (Materials and Methods). The "cell number" in each case refers to the number of these cells plated to generate the SA activity. "MC for adherence" refers to the MC plated for the 1.5-h adherence step (Materials and Methods) and "cell number" in this case means the number of these cells plated to generate the adherent cells. Solid columns: CM from the various cell populations were prepared in $\alpha$-MEM + 5% FBS from 24-h cultures. Hatched and stippled columns: CM were prepared in the same way but in $\alpha$-MEM + 5% FBS supplemented with 10 $\mu$g/ml T12 CWS or T6 CWS, respectively (see Materials and Methods). The mean fibrinolytic activities in the wells containing media or 3 $\mu$g/ml T12 CWS or 3 $\mu$g/ml T6 CWS, as controls were all 0.2% (data not shown). Open columns: lysozyme was also measured in the CM prepared in $\alpha$-MEM + 5% FBS; the values refer to the mean total enzyme activity in the duplicate cultures.

separate experiments. The MDP sample was active in both serum-free and in FBS-containing medium even at concentrations as low as 0.1 ng/ml. In the same experiments, >1 $\mu$g/ml T6 CWS was required to elicit SA activity, but the maximum SA activity that could be generated from optimal concentrations of MDP and T6 CWS was similar (data not shown). Thus, the arthritogenic MDP structure is also active in the in vitro model system under study.

*Cell Number and SA Activity.*   In Fig. 4, the relationship of PBMC number to SA activity resulting from T6 CWS action is plotted. SA activity can still be detected from 5 × 10$^5$/ml PBMC and is still increasing at a cell concentration of 4 × 10$^6$/ml $\alpha$-MEM + 5% FBS. A similar cell dose response is evident in serum-free medium or, if the stimulus is either 1 $\mu$g/ml MDP or 10 $\mu$g/ml Con A, in serum-free or in FBS-containing medium (data not shown).

*Cell Type and SA Activity.*   As mentioned above (Figs. 2 and 3), adherent cells from PBMC could elicit SA activity in response to streptococcal CWS and MDP. Fig. 5 shows more data in which total PBMC, adherent cells, and the nonadherent, nonphagocytic (i.e., iron carbonyl-treated) populations (see Materials and Methods) are compared as sources of SA activity in response to FBS, T12 CWS, and T6 CWS in the same experiment. The number of adherent cells obtained by this procedure is of the order of 10–20% of the total MC population. As can be noted, the adherent cell population was the most active on a per cell basis as a source of SA activity. As monocyte-macrophages are the predominant cell type present in the adherent cell cultures derived from the PBMC, these data would suggest that this cell class is responsible for the SA activity. Supporting this statement are the lysozyme levels monitored in the various CM from the FBS-containing cultures. Lysozyme is a monocyte-macrophage product (34) and the active CM for SA activity are generally

those which arise from a cell population with the higher levels of lysozyme in the control (i.e., FBS) cultures.

This type of experiment has been repeated with >20 donors. In several experiments, the nonadherent cells (even sometimes after subsequent phagocytosis of iron carbonyl) had appreciable SA activity, and this was particularly noticeable when Con A or PHA was the stimulus. However, when these same CM were assayed for lysozyme activity, then it was found that the most potent SA-containing CM had the most lysozyme. These data suggest that the number of monocyte-macrophage-type cells present in a nonadherent population can be quite variable and in some cases can comprise an appreciable percentage of the original number. This observation has been made by others (35) and can depend on variables such as the PBMC donor and the serum used to attach the cells. For the adherent cells themselves, it is difficult to exclude the presence of a few contaminating, nonmonocyte, adherent cells that could be contributing to the SA levels. SA activity could also be elicited from T6 CWS-treated adherent cells after a 24-h adherence of PMBC (data not shown).

As an alternative approach, PBMC were enriched for T lymphocytes $(T^+)$ and screened together with the T lymphocyte-depleted cells $(T^-)$. The data in Fig. 6 demonstrate that $T^+$ cells were poor producers of SA activity, with the $T^-$ cells being enriched for both SA and lysozyme activity. The adherent cells were again active both from the total PBMC (Fig. 6) and when obtained in turn from the $T^-$ population (data not shown). In a separate experiment, if B lymphocytes were depleted directly from the PBMC (Materials and Methods), then no loss of SA activity with 5% FBS, T6 CWS, and Con A occurred on a per cell basis (data not shown). The results in Fig. 6 are consistent with those of Fig. 5 and suggest that cells belonging to the monocyte-macrophage subclass can be directly responsible for the SA activity.

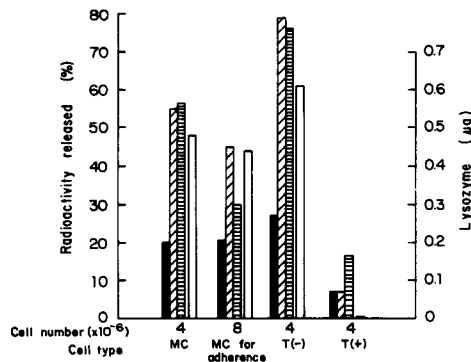*Kinetics of SA Generation.* The rate of appearance of the SA activity from adherent



Fig. 6. T cell depletion and SA activity. Human synovial fibroblasts (1st passage) from a normal donor (R.W.) were plated in $^{125}$I-fibrin coated wells, and the PA activity measured after 24 h in the presence of the different MCCM preparations (Materials and Methods). "MC" refers to the total PBMC population; as before, "MC for adherence" indicates MC plated for a 1.5-h adherence; "$T^-$" and "$T^+$" refer to T lymphocyte-depleted and -enriched populations, respectively (Materials and Methods). Solid columns: CM from the various cell populations were prepared in $\alpha$-MEM + 5% FBS from 24-h cultures. Hatched and horizontal columns: CM were prepared in the same way but in $\alpha$-MEM + 5% FBS supplemented with 10 $\mu$g/ml T12 CWS or 10 $\mu$g/ml Con A, respectively (see Materials and Methods). The mean fibrinolytic activities in the wells containing media, 3 $\mu$g/ml T12 CWS, or 3 $\mu$g/ml Con A as controls were ~7% (data not shown). Open columns: lysozyme was also measured in the CM prepared in $\alpha$-MEM + 5% FBS; the values refer to the mean total enzyme activity in the duplicate cultures.

cells in α-MEM + 5% FBS in response to T6 CWS is presented in Fig. 7. Increased SA activity was easily detectable at 12 h, with plateau levels reached by 48 h. Similar kinetics were observed in α-MEM + 5% FBS and also with Con A (5 μg/ml) in α-MEM + 5% FBS. With some cultures, plateau levels were reached within 24 h. SA levels at earlier time points and monocyte-macrophage metabolic requirements for the formation of the SA activity are currently under study.

*Properties of SA Activity.*    In this section, preliminary data from experiments designed to characterize the SA activity are included.

Gel chromatography.    The SA activity in MCCM prepared in α-MEM alone or in α-MEM containing 5 μg/ml Con A was completely retained, and therefore could be concentrated by ultrafiltration with an Amicon UM-10 membrane (Amicon Corp., Scientific Systems Div., Lexington, MA). The concentrated CM from Con A-containing cultures was then applied to a Sephadex G-100 column, and the SA activity appeared in the range of 28,000–34,000 mol wt. (Fig. 8).

Seeing that both IL-1 and IL-2 have been found in the CM from PBMC (29, 31), the ability of the various fractions from this column to stimulate [$^3$H]TdR incorporation with mouse thymocyte cultures was monitored (see Materials and Methods). The main peak of thymocyte-stimulating activity was at ~20,000 mol wt and was clearly separate from the SA activity (data not shown, but see Fig. 9). The thymocyte assay does not distinguish between IL-1 and IL-2 (36).

Several stimuli, such as antigens (37) and mitogens (38, 39), are able to act as inducers, from T lymphocytes and/or macrophages, of a subclass of IFN known as IFN-γ. Another potent inducer of IFN-γ is SEB (28). In Fig. 9, the IFN-γ and SA activity profiles after Sephadex G-100 chromatography for leukocyte CM prepared in the presence of FBS and SEB are provided. The SA activity is again in the range of 25,000–34,000 mol wt with the molecular weight of the IFN-γ being ~38,000–45,000 (28). Thus the molecular weight range for the SA activity in serum-free medium (Fig. 8) is similar to that in FBS-containing medium (Fig. 9). The fact that IFN-γ is unstable (39), whereas SA is stable (see below) if stored overnight at pH 2 at 4°C, is
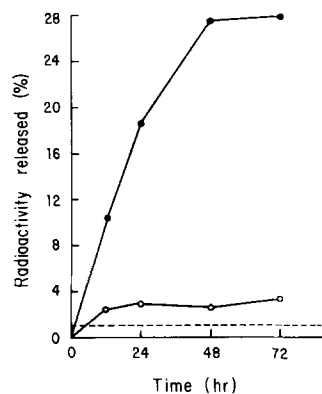


Time (hr)

Fig. 7.    Kinetics of appearance of SA activity. This was the same experiment as in Fig. 4, with the MCCM being harvested at different time points and a constant number of the same MC population (4 × 10$^6$) used to prepare the CM. MCCM prepared in 1 ml α-MEM + 5% FBS (O); MCCM prepared in the same way but in 1 ml α-MEM + 5% FBS containing 30 μg/ml T6 CWS (●) (Materials and Methods). The mean fibrinolytic activities in the wells containing media or 10 μg/ml T6 CWS as controls were 1% (– – –).
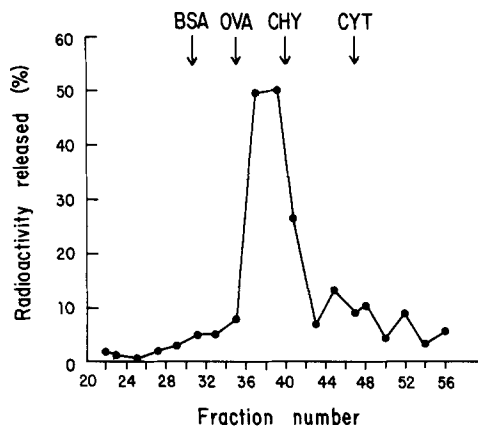
Fig. 8. Purification of SA activity by gel chromatography. Human synovial fibroblasts (3rd passage) from an osteoarthritic donor (L.F.) were plated in $^{125}$I-fibrin coated wells, and the PA activity measured after 48 h in the presence of the different column fractions (40-μl) (see Materials and Methods). The cultures were also supplemented with 50 μg/ml gentamycin. The fractions for assay were obtained as follows. The 24-h serum-free CM (200 ml) prepared by stimulation of 4 × 10$^6$ PBMC/ml with 5 μg/ml Con A (Materials and Methods) was concentrated at 4°C to 10 ml using an Amicon UM-10 ultrafiltration membrane. 8 ml of this concentrated sample was loaded onto a 2.6 cm × 78 cm Sephadex G-100 column equilibrated with 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.2. 7-ml fractions were collected every 45 min; absorbance at 280 nm was recorded during the run. The absorbance was ~0.03 U near the void volume (fraction 18) and background values for the remaining fractions. Molecular weight standards were run consecutively under the same conditions (bovine serum albumin (BSA), 67,000; ovalbumin, (OVA) 43,000; chymotrypsinogen A (CHY), 25,000; cytochrome C (CYT), 12,500). Each fraction was assayed in duplicate; the linearity of the response for each fraction was checked by monitoring fourfold dilutions of the fractions. The mean fibrinolytic activities in the wells containing the column buffer and in the starting CM (diluted 1:20) as controls were 1.5% and 13%, respectively.

consistent with this chromatographic separation. The effect of the SEB on the generation of SA activity has not yet been studied, and the SA activity present in the experiment may have been due to stimulation by the FBS.

The IL-2 activity profile was also monitored, with the main activity at ~18,000–25,000 mol wt, and again being delineated from the peak of SA activity (Fig. 9). In support of this preliminary biochemical distinction, it is perhaps worth noting that human IL-2 is generally considered to be a T lymphocyte product (40) although monocyte-macrophages appear to regulate its production (41).

SA ACTIVITY IN PURIFIED IL-1. IL-1 from monocytes has been implicated as a stimulator of the collagenase levels of adherent human rheumatoid synovial cells (42, 43). Also, LPS-treated adherent cells release IL-1 into the CM (44). We have found as well that the adherent cells release IL-1 in response to T12 CWS (data not shown). However, human IL-1 has a molecular weight of ~13,000 by gel chromatography (31). This would suggest that SA and IL-1 were distinct entities. In Table I, the SA activities of IL-1 preparations of different degrees of purity are compared. It is apparent that as the specific activity of the IL-1-containing samples increased (Stage I to Stage III) their SA activity declined, with the most highly purified IL-1 tested (Stage III) having only weak SA activity.

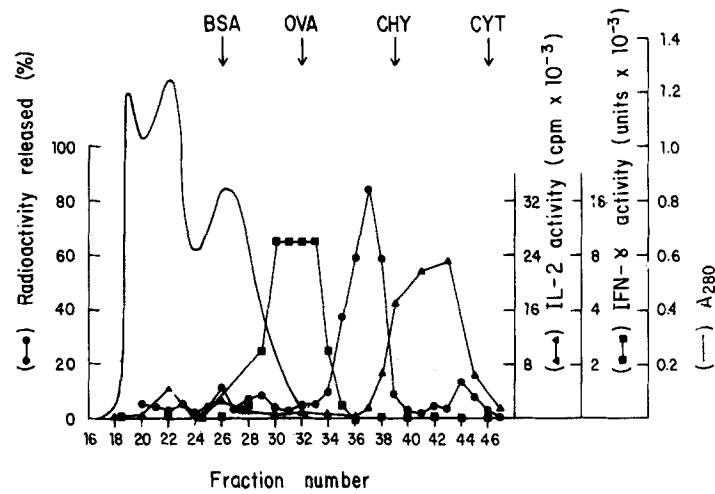STABILITY OF THE SA ACTIVITY. The pooled and concentrated peak of SA activity

Fig. 9. Separation of SA activity from IFN-γ and IL-2 activities by gel chromatography. Human synovial fibroblasts (6th passage) from an osteoarthritic donor (E.P.) were plated in $^{125}$I-fibrin coated wells, and the PA activity measured after 48 h in the presence of the different column fractions as in Fig. 8. The fractions for SA assay were obtained as follows. IFN-γ-containing CM was prepared by culturing peripheral blood leukocytes at $1 \times 10^6$ cells/ml RPMI + 2% FBS for 72 h in the presence of 0.02 μg/ml SEB (28). The CM was partially purified by chromatography on controlled-pore glass and IFN-γ containing fractions pooled and lyophilised (28, 39). The sample (5 ml) was then applied to a Sephadex G-100 column (2.6 cm $\times$ 50 cm) equilibrated with 20 mM sodium phosphate, 0.15 M NaCl, pH 7 2; 5 ml fractions were collected at a flow rate of 5 min/ml. Absorbance at 280 nm was recorded during the run. The molecular weight standards were as in Fig. 8. The mean fibrinolytic activity in the wells containing the column buffer as control was 4.2%. IFN-γ and IL2 activities were quantitated as in Materials and Methods.

TABLE I

SA Activity in IL-1 Samples of Different Degrees of Purity

| Degree of purification of IL-1 sample | IL-1 activity added | Fibrinolysis |
|---|---|---|
| | $U$ | % |
| Stage I | 0.8 | 8 |
| (10–50,000-mol wt fraction) | 3.2 | 17 |
| | | |
| Stage II | 1.0 | 5 |
| (IEF-purified) | 4.0 | 7 |
| | | |
| Stage III | 0.9 | 3 |
| (PAGE purified) | 3.6 | 5 |
| | | |
| Buffer (PBS) | — | 3 |

Human synovial fibroblasts (6th passage), from an osteoarthritic donor (L.F.), were plated in $^{125}$I-fibrin-coated wells (Materials and Methods). After 48 h, the cultures were washed twice with α-MEM and placed in α-MEM + 5% ATFBS containing two concentrations of each of the three different IL-1 preparations (Materials and Methods). Aliquots of the supernatant medium were removed from the duplicate cultures at 24 h and assayed for solubilized radioactivity as a measure of the fibrinolytic activity.

from the Sephadex G-100 column (Fig. 8) (see Materials and Methods) was used for the following tests:

(a) Temperature and pH.   The SA activity was stable at 4°C and 37°C for 24 h, only partially destroyed by 70°C after 1 h and boiling for 2 min, but completely destroyed by boiling for 10 min. In addition, there was only slight loss of activity after three cycles of freezing at −70°C and thawing. No loss of SA activity was observed at pH 2–10 overnight at 4°C.

(b) 2-Mercaptoethanol.   SA activity was retained after incubation for 10 min at 37°C with 0.1 M 2-mercaptoethanol, followed by dialysis.

(c) Enzyme treatment.   After incubation with trypsin (Trypsin-TPCK, Worthington Biochemical Corp., Freehold, NJ) (100 µg/ml, 0.1 M Tris, pH 8.1) for 1 h at 37°C, ~70% of the SA activity remained, whereas for α-chymotrypsin (Sigma Chemical Co., St. Louis, MO) (1 mg/ml, 0.1 M Tris, pH 8.1) only ~25% of the activity was left. SA does not seem to be a serine protease, as phenylmethylsulfonyl fluoride did not block its activity (11). The activity was stable after treatment with neuraminidase (0.1 U/ml, 1 M acetate buffer, pH 5.0) for 1 h at 37°C. These treatments suggest that the SA activity is protein in nature without functionally important sialic acid residues although, of course, the sialic acid residues may be inaccessible to the neuraminidase action. More recent results indicate that the SA activity does not bind to a Con A-Sepharose affinity column (data not shown).

## Discussion

In a previous publication (11), it was found that PBMC, particularly after Con A treatment, liberated an activity that increased the PA activity of passaged, nonrheumatoid human synovial fibroblasts. We have demonstrated above that MDP and CWS preparations from group A streptococci are capable of eliciting the SA activity. There are other in vitro systems (see below) that study the MC modulation of synovial cell function as models for RA, but this is the first report in which an arthritogenic agent (and one that has been implicated in human disease [2–5]) has been shown to potentiate the MC effect.

It should be noted, however, that with a few PBMC populations, almost maximum SA activity was achieved in the absence of any exogenous stimulus. FBS (>1%), even though having inhibitors that can block the PA activity during assay (20), is generally stimulatory by itself (Fig. 1), and in some cases very little increase above the FBS value is attained with any of the agents tested. In other words, dramatic increases can be obtained in most cases with CWS, Con A, and PHA, but sometimes the serum-free or FBS-containing control cultures can also have significant activity. As mentioned, LPS is stimulatory and could be present in some of the sample solutions being tested for stimulatory activity (44); however, this does not seem to be generally the case, as preincubation at room temperature for 3 h of the CWS, Con A, PHA, and FBS stock solutions with 50 µg/ml polymyxin B (44) did not reverse their effects, although it successfully reversed the LPS effect for doses of LPS ≤100 ng/ml.

Although culture conditions were made as reproducible as possible in the present study, different synovial fibroblast lines at various passage numbers were used as target cells for the monitoring of the SA activity. This approach permits an evaluation to be made of the extent of the variation in the system due to nature of the synovial donor; however, it should also be borne in mind that the PA activities from other cell

types have been demonstrated to be under the control of a number of variables, including serum growth rate and cell density (45). Some of these variables have recently been discussed with particular reference to human synovial fibroblast lines.[2] For these reasons, the absolute PA activities should not be compared between different experiments, but rather the relative activities within an experiment considered.

As mentioned in Results, the data are consistent with the monocyte-macrophage cell class being the cells responsible for SA activity. In addition, several workers have strong evidence that both streptococcal peptidoglycans and MDP can act directly on monocyte-macrophages to alter both cellular function and product formation (for example see refs. 46–48). However, a few residual lymphocytes could be contributing to the SA levels, and there might also be some cellular interactions between lymphocyte and macrophages. These questions might best be answered by enrichment and depletion of cell populations with monoclonal antibodies specific for PBMC subpopulations, which could enable a more definitive separation of the responsible cell types.

The observation that MDP could generate SA activity suggests that it is the active principle in the CWS, a finding consistent with previous studies on the biological properties of streptococcal and other bacterial cell wall preparations (32, 33). This result would suggest that phagocytosis might not be the primary trigger for SA generation by the CWS samples, but rather that there might be a critical interaction of the peptidoglycan moiety with the monocyte-macrophage membrane. In this regard, it has been found that readily phagocytized latex particles ($1$-$\mu$m Diam) were poor stimulators of SA activity (unpublished observations) and, as mentioned above, there were several active agents, namely PHA, Con A, and LPS, which were presumably functioning independently of a phagocytic mechanism.

Other preliminary results have shown that streptococcal membrane preparations (obtained as in ref. 49) are as active, on a direct-weight basis, as the corresponding CWS preparation in releasing SA activity from the PBMC (unpublished observations). Because the bacterial membrane samples contain very low amounts of peptidoglycan (49), other bacterial components can be stimulatory besides the peptidoglycan moiety. However, the importance of the CWS stimulation may be in the observation that it is these structures and not the membrane that cause experimental arthritis (7). The difference in biological activity may be due to the known persistence (nonbiodegradability) of cell walls (6, 7) when compared with membranes. Current studies are directed towards determining both the range of streptococcal groups and other bacterial strains that are active in the system.

On the basis of biochemical data, it has been proposed that the mononuclear cell factor, which elevates the latent collagenase activity of rheumatoid cell cultures, is identical to IL-1 (42, 43); the preliminary biochemical evidence above indicates that SA activity(ies) is distinct from IL-1, IL-2, and IFN-$\gamma$. The molecular weight estimate from gel chromatography ($\simeq$30,000) may be, of course, the result of aggregation or association with contaminant proteins; dissociating conditions may yield a lower value. Also, it cannot be assumed that the SA activity(ies) prepared under different culture conditions and with different stimuli will always have identical biochemical properties. The SA would seem to be different also from connective tissue activating peptide I, which is extracted from human spleen cells (50). This molecule stimulates hyaluronic acid production from human synovial fibroblasts but by Sephadex G-100 chromatography has a molecular weight of $\sim$15,000 (50). But again, more detailed

biochemical analysis is necessary before the nonidentity can be stated with certainty, particularly as crude MCCM also stimulate hyaluronic acid levels (data not shown).

We would like to suggest that the in vitro human model system described in the present paper has several features consistent with those of the rat arthritis model established by intraperitoneal injection of group A streptococci CWS (7, 8). Histologic features of this arthritis included an acute exudative phase followed by an erosive synovitis that led to destruction of cartilage and subchondral bone, and fibrous ankylosis of the joints. The responsible moiety seemed to be a peptidoglycan-polysaccharide fragment of the cell well that persisted in tissue, with much of this material localized within macrophages (7, 8). We have shown above that group A streptococcal CWS interact with monocyte-macrophages to elicit an activity capable of stimulating synovial cells to elaborate, in turn, a protease system (PA/plasmin) capable of contributing to some of the invasive, destructive, and inflammatory processes in an arthritic joint (see introduction). Seeing that high PA activity often results after oncogenic transformation of fibroblasts (20), it was proposed previously (11) that the mononuclear cell-mediated change in the synovial cell metabolism is consistent with some of the morphological descriptions of the pannus as "tumor-like" (51, 52) and also "aggressive and, in a sense, malignant," (53). It is not our intention to minimize the possible direct role of inflammatory cells themselves in the destructive and inflammatory events in the RA joint, but we propose that the modified synovial fibroblast cell could also be important (see also refs. 23, 52).

It would seem imperative in future studies to search for the presence of the SA activity in rheumatoid fluids, in CM from tissue, and also in animal model systems as a test of the relevance of the above proposal to disease. Future work will also examine the possible involvement of lymphocyte-macrophage interaction in the elicitation of SA activity, particularly as such an interaction involving streptococcal cell wall antigen presentation might be relevant to an understanding of the likely genetic component of RA (54).

## Summary

Group A streptococcal peptidoglycan has previously been shown to be arthritogenic in rats and has been implicated as a structure present in a class of possible etiologic agents for rheumatoid arthritis. The present study reports that conditioned medium from human monocytes, after interaction with cell wall sonicates of four group A streptococcal strains, stimulates the plasminogen activator (PA) activity of nonrheumatoid synovial fibroblasts. Low concentrations of $N$-acetylmuramyl-L-alanyl-D isoglutamine (muramyl dipeptide) can also generate this synovial activator (SA) activity from human monocytes. Preliminary biochemical data suggest that the SA activity is distinct from interferon-$\gamma$, interleukin 1, and interleukin 2. These results indicate that agents that are arthritogenic in rats can modulate human synovial fibroblast functions via monocytes. The findings are proposed to have possible significance for an understanding of the cellular interactions involved in the formation and function of the rheumatoid pannus, because PA has been invoked as possibly being generally important for the processes of cell migration, tissue remodeling, and inflammation.

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