IFNγ, suggesting that more than one, independently-regulated ELAM may be involved. Cytokine effects require *de novo* protein synthesis. The T cell ligand is not yet known. These cytokines do not enhance the adhesiveness of the *lymphocytic* partner, and MAbs to the common β subunit of LFA-1, CR3 and p150,95 fail to reduce cytokine-induced T cell-endothelial cell adherence.

(iii) $4B4^+$ cells (enhanced CD4/CDw29 expression: helper-inducer subset) are much more numerous than the suppressor-inducer subset in rheumatoid synovial fluid or synovium, and they show an enhanced tendency to bind to unstimulated or IL1-stimulated endothelial cells. Proliferating T cells are predominantly 4B4 positive, and these cells tend to aggregate in the absence of endothelial cell partners. These observations strongly suggest that the $4B4^+$ T cell subset, which is thought to consist largely of memory cells, has developed surface molecules during sensitization: these facilitate adhesion to endothelium and emigration into non-lymphoid tissues.

(iv) Molecules conveying organ specificity of T-lymphocyte trafficking (see below).

The opportunity for modulating chronic inflammatory diseases by inhibiting T cell-endothelial cell interactions *via* any or all of these mechanisms was raised, with the encouragement offered, for example, by the proven efficacy of thoracic duct drainage or leucopheresis in severe rheumatoid arthritis.

Dr. Tony Freemont (University of Manchester) described, and elegantly illustrated, the unusual 'plump' morphology of the cells in the high endothelial venules (HEV) that develop after up to 6–8 weeks of chronic lymphocytic trafficking at a site of inflammation. These HEV cells are very similar to those normally occurring in the lymph nodes, where lymphocytic migration is an essential event in healthy individuals, and they are both morphologically and histochemically distinct from other endothelial cells. Initial lymphocytic infiltration may induce, *via* local IL1 production, the development of HEV, but Dr. Freemont has been unable to induce HEV morphology by IL1, IFNγ, etc. Both T and B lymphocytes can migrate through HEV endothelial cells, but only T cells have the aryl sulphatases and glycosidases necessary to migrate further, through the distinctive trilaminar, pale perivascular sheath laid down by HEV cells. This sheath contains substantial quantities of chondroitin and keratan sulphates, so that HEV cells can also be distinguished by their high rate of $^{35}SO_4$ uptake.

Dr. Freemont suggested that the perivascular sheath served to allow continuous lymphocytic trafficking while preventing the oedema that would normally occur through concomitant fluid extravasation.

Adhesion of lymphocytes to (HEV) endothelial cells is, as discussed by the other speakers, a major component of lymphocyte trafficking, and the HEV cells clearly express molecules enhancing this adhesion. Perhaps of greatest interest is the evidence that the molecular basis of this phenomenon may show organ-selectivity. Thus, the MAb MEL14 reduces adhesion to cervical HEV, but fails to inhibit lymphocytic adhesion to the endothelial cells in Peyer's patches or rheumatoid synovia. Adherence to mesenteric endothelial cells was partially inhibited. The potential for elegant pharmacological interventions is clear, though a great deal more fundamental work is required.

lytes either adhered to the bacterial surfaces and neutralized the polycations or that these polyanions inactivated certain of the autolytic wall enzymes.

Since a variety of reactive oxygen species [17–21] and proteolytic enzymes are capbable of degrading a variety of macromolecular substances, it was of interest to explore the possibility that the inability of leukocytes to degrade microbial cell wall components in inflammatory sites might be linked with the selective inactivation of their autolytic wall enzymes by reactive oxygen species and by proteinases. This might contribute to the perpetuation and propagation of chronic inflammatory sequelae.

The present communication shows that bacteriolysis *in vitro* induced by cationic agents in markedly depressed by exposing bacteria either to reactive oxygen species or to proteinases. The possible role of scavengers of oxygen radicals and proteinases inhibitors in the facilitation of cell wall degradation *in vivo* is discussed.

Materials and methods

Radiolabeled staph. aureus

Staphylococci (strain Haus) which had been kindly supplied by the Robert Koch Inst. West Berlin, were cultivated in brain heart infusion broth (Difco) which contained 0.25 μCi/ml of ^{14}C-N-acetylglucosamine specific activity of 55 mCi/mmol Amersham) and harvested from the logarithmic phase of growth. The labeled cells were washed in saline buffered with 0.01 *M* phosphate pH 7.4 (PBS). Bacterial suspensions containing approximately 15 000 cpm/ml (approx. 2×10^7 organisms/ml) were employed.

Induction of bacteriolysis

One ml aliquots of radiolabeled bacteria were incubated for various time intervals either with egg-white lysozyme (LYZ) (10–250 μg/ml) (Sigma Chemical Co., St. Louis, Mo, USA), with a crude preparation of leukocyte cationic proteins (LCP) isolated from human neutrophils as described [9], or with freeze and thaw extract of human neutrophils (ENZ) which contained approx. 10 mg protein/ml. All these agents have been found to initiate cell wall lysis in staphylococci [7, 8]. Following incubation at 37 °C for various time intervals, the tubes were centrifuged at 2000 *g* for 10 min and the percentage of solubilized radioactivity was determined in the supernatant fluids employing a Kontron scintillation counter as described [8].

Effect of H_2O_2 and proteases on bacteriolysis

Radio-labeled staphylococci were first incubated for various time intervals at 37 °C with 1) hydrogen peroxide (10–1000 nmoles/ml); 2) with a mixture of xanthine (200 μg/ml)-xanthine oxidase (3 units/ml) (X-XO); 3) with glucose (0.01 *M*) and glucose oxidase (0.4 units/ml). Both systems are known to generate large amounts of hydrogen peroxide; 4) with pronase (200 μg/ml); 5) with crystalline trypsin (200 μg/ml); and 6) with ENZ (100 μg protein/ml) (see above). In some experiments catalase (1000 units/ml), soybean trypsin inhibitor (SBTI) (500 μg/ml) or phenyl methyl sulfonyl fluoride (PMSF) (200 μg/ml) were also included in the reaction mixtures (see results). These were added to inhibit either the oxygen radicals or the proteinases. All the chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., USA The pretreated bacterial cells were then further incubated for 15 hrs at 37 °C either with LYZ, with LCP or with ENZ (inducers of bacteriolysis), and the percentage of radioactivity solubilized was determined as described above. In some experiments we have also tested the effect of hydrogen peroxide and of proteinases on lysis of staphylococci by lysostaphin [22] (a mixture of cell wall lysing enzymes – Sigma). We have either preincubated the lysostaphin preparation with the various inhibitory agents or added the lysostaphin and the inhibitors simultaneously.

Results

Effect of H_2O_2 on bacteriolysis

Figure 1 shows that the exposure of staphylococci to hydrogen peroxide for 60 min at 37 °C inhibited in a dose-dependent fashion the breakdown of the labeled cell walls which was initiated by lysozyme. The inhibitory effect of hydrogen peroxide on bacteriolysis was, hower, totally reversed by catalase. Catalase, however, failed to reverse this inhibition if added together with the activators of autolysis, suggesting that hydrogen peroxide had to be prein-

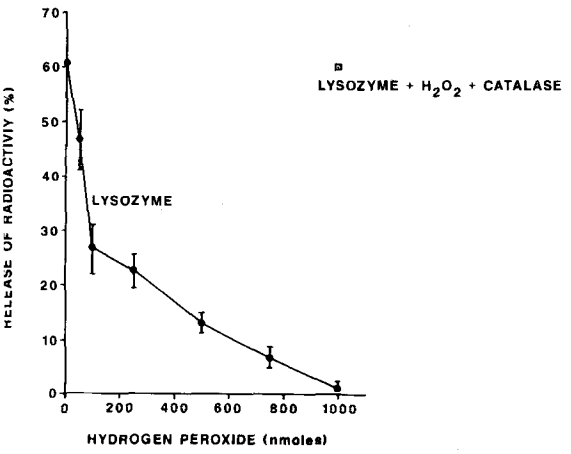

Figure 1
The effect of hydrogen peroxide on bacteriolysis. Radiolabeled staphylococci were exposed for 60 min. at 37 °C to hydrogen peroxide or to peroxide in the presence of catalase (100 U/ml) and then washed in saline. Egg white lysozyme (LYZ) (250 μg/ml) an activator of autolysis was then added and the solubilization of radioactivity was determined in the supernatant fluids after incubation at 37 °C for 15 hours. Bars are ranges of the value obtained for 4 separate experiments.

cubated with the bacterial cells in order to exert its inhibitory effect on bacteriolysis. It is of note that although staphylococci are catalase positive, the amounts of staphylococci employed in the assays did not significantly reduce H_2O_2 activity. This precluded the employment of a catalase inhibitor (i.e. sodium azide) which might have interfered with the action of added catalase. Further studies with a hydrogen peroxide-generating systems, e.g. xanthine-xanthine oxidase (Table 1) yielded similar results, employing leukocyte extracts as an activator of autolysis.

Since hydrogen peroxide might have acted by destroying the autolytic wall enzymes, we also tested the effect of large amounts of this agent on lysis of staphylococci with lysostaphin. In this experiment the lysostaphin preparation was first exposed to hydrogen peroxide (1000 nmoles/ml) then catalase, in amounts sufficient to decompose all the hydrogen peroxide, was added followed by the addition of staphylococci. Surprisingly, hydrogen peroxide failed to inactivate lysostaphin action. It appears, therefore, that either the effect of hydrogen peroxide on the whole bacterial cell acted via a different mechanism, or that the commercially

Table 1
Effect of xanthine-xanthine oxidase (Xan-Xan Oxi) on bacteriolysis induced by leukocyte extracts (ENZ).

Radiolabeled bacteria preincubated with	Followed by	Percent release of radioactivity*
None	None	15.0
None	ENZ	78.0
Xan-Xan Oxi	None	11.0
Xan-Xan Oxi	ENZ	29.0

* The data are the mean percent release of 4 experiments performed in triplicates. Radiolabeled staphylococci suspended in PBS pH 7.4 plus NaN_3 (10 m*M*) were preincubated for 2 hrs at 37 °C with xanthine (200 μg/ml) and xanthine oxidase (3 units/ml). This mixture yielded approximately 500 nmoles of hydrogen peroxide in 2 hrs. The treated cells were then further incubated for 15 hrs with a selected lytic preparation of leukocyte extract (see above) (ENZ) containing approximately 100 μg protein/ml.

viable lysostaphin preparation might not represent the ture indigenous autolytic wall enzymes of this staphylococcal strain.

Effect of proteolytic enzymes on bacteriolysis

Previous studies have shown that crude extracts of human blood leukocytes (ENZ) containing cationic proteins, and a more purified cationic protein (LCP) preparation induced cell wall lysis in staphylococci [7, 8]. We have, however, also observed that not all the ENZ or LCP preparations had lytic effects when employed at high protein concentrations [7]. Furthermore, some of the ENZ and LCP preparations depressed the spontaneous release of radioactivity from staphylococci incubated in buffers alone (spontaneous autolysis) due to cell-wall turnover. These findings suggested that the ENZ and LCP might contain 2 types of agents. An activator of autolysis (presumably cationic proteins) and an inhibitory materials. The possibility that the inhibitory agent present in these preparations was associated with proteinases, which could inactivate the autolytic wall enzymes, was investigated. Table 2 shows that when a non-lytic preparation of ENZ was mixed with proteinase inhibitors (SBTI, PMSF), it readily induced bacteriolysis. This occurred even with large amounts of the fraction. It was, therefore, suggested that its high proteinase content prevented bacteriolysis. Table 3 shows that both trypsin and pronase markedly suppressed LYZ-induced bacteriolysis and

Table 2. Effect of leukocyte extracts (ENZ), proteinase inhibitors and lysozyme (LYZ) on bacteriolysis.

Radiolabeled staphylococci pretreated with	Followed by	Percent release of radioactivity*
Leuk. extract (ENZ)	None	15.0
ENZ + PMSF (200 μg/ml)	None	61.0
ENZ + SBTI (500 μg/ml)	None	60.0
ENZ	LYZ	31.0
ENZ + PMSF	LYZ	86.0
ENZ + SBTI	LYZ	88.0
None	LYZ	65.0

* The data are the mean percent release of 5 experiments. Radiolabeled staphylococci were preincubated for 60 min at 37 °C with a selected non-lytic preparation of ENZ (500 μg protein/ml) (see text), or with ENZ in the presence of soybean trypsin inhibitor (SBTI) of phenyl methyl sulfonyl fluoride (PMSF). The cells were then challenged with LYZ (250 μg/ml) which acted as an activator of autolysis.

Table 3
Effect of proteinases on bacteriolysis induced by LYZ.

Radiolabeled staphylococci pretreated with	Followed by	Percent release of radioactivity*
None	None	10.0
Pronase (100 μg/ml)	None	5.0
Pronase + PMSF (200 μg/ml)	None	12.0
None	LYZ (250 μg/ml)	74.0
Pronase	LYZ	28.0
Pronase + PMSF	LYZ	73.0
Trypsin (200 μg/ml)	None	8.0
Trypsin + PMSF	None	25.0
Trypsin + SBTI (500 μg/ml)	None	11.0
Trypsin	LYZ	32.0
Trypsin + PMSF	LYZ	98.0
Trypsin + SBTI	LYZ	85.0

* The data are the mean percent release of 3 experiments. Radiolabeled staphylococci were preincubated for 60 min at 37 °C either in buffer alone, in buffer containing either proteinases or a mixture of proteinases and their inhibitors. The treated cells were then challenged with lysozyme to activate autolysis.

Table 4
Effect of pronase on lysostaphin activity.

Lysostaphin (5 units/ml) pretreated with	Percent release of radioactivity*
None	99.0
Pronase (200 μg/ml)	12.5
Pronase + PMSF (200 μg/ml)	97.0
PMSF	99.0

* The data are the mean percent release of 4 experiments.

that proteinase inhibitors totally reversed the proteinase effects, suggesting the inactivation of the autolytic wall enzymes by the proteinases. To further test this assumption we treated lysostaphin either with pronase or with a mixture of pronase and PMSF. Radiolabeled staphylococci were then added, and the degree of cell lysis was determined. Table 4 shows that pronase totally destroyed the bacteriolytic effect of lysostaphin, and that PMSF reversed the inhibition of pronase on bacteriolysis.

Discussion

The *in vitro* studies showing that leukocytes and inflammatory exudates contribute bactericidal cationic proteins [2–4, 7, 8] which might also act as activators of the autolytic wall enzymes of bacteria leading to their demise (suicide), and the finding that lysozyme might functions not only as a muramidase but also as an activator of autolysis [8–10] shed new light on the role played by leukocytes in bacteriolysis.

The rich polyanionic content of inflammatory exudates and the ability of macrophages to pinocytose polyanions was considered in our earlier publications as one of the major factors which contributed to the persistence of undegraded cell wall components in tissue lesions [7, 8, 16].

However, the additional findings reported here that both hydrogen peroxide and proteinases also inhibit autolysis of staphylococci (Fig. 1 and Tables 2–4) and the report that reactive oxygen species might enhance the digestion of *E. coli* proteins [21] further complicate our understanding of how microbial cell wall components are degraded by leukocytes *in vivo* [11–15].

It thus appears that a delicate balance between activators of autolysis (cationic polyelectrolytes) and inhibitors of autolysis (anionic polyelectrolytes, hydrogen peroxide, proteinases) might determine the fate of microbial cell wall components within phagocytes in inflammatory sites. To overcome the destruction of the autolytic wall enzymes of bacteria by activated leukocytes *in vivo*, it might be speculated that scavangers of reactive oxygen species and proteinase inhibitors encased in liposomes, can perhaps be effectively employed to counteract the inhibitors of autolysis. Since mutanolysin (an enzyme capable of degrading cell wall components of streptococci) when injected to animals markedly inhibited the development of

chronic erosive arthritis induced by streptococcal cell walls [23], and since a series of cell wall degrading enzymes active against other microbial species are also available [24] it is speculated that such agents might be employed to combat the persistence of highly-phlogistic microbial cell wall components in tissues. Furthermore, the availability of scavangers of reactive oxygen species with prolonged action *in vivo* [25, 26] also suggest that treatment of post-infectious sequelae induced by microbial cell wall components, might be dealt with therapeutically [27]. Further studies on the effect of reactive oxygen species and proteinases derived by activated leukocytes on the survival of bacterial cell walls *in vivo* is underway.

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