CYTOLOGICAL STUDIES IN ASSOCIATION WITH LOCAL IN-JECTIONS OF STREPTOKINASE–STREPTODORNASE INTO PATIENTS [1]

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The use of streptokinase [2] and streptococcal desoxyribonuclease [2] as therapeutic agents has been the subject of recent reports from this clinic and laboratory (1, 2, 3). The injection of these enzymes into areas of inflammatory exudation has been observed (1) to be accompanied by a local outpouring of leukocytes. A striking decrease in the number of degenerated leukocytes as well as a rapid disappearance of extracellular desoxyribose nucleoprotein has also been described. These changes have been attributed to the action of the streptococcal desoxyribonuclease (2).

In order to obtain a more complete understanding of the effects of these lytic enzymes, the cytological and cytochemical reactions evoked locally have been studied in some detail, together with direct observations of the viability, motility and phagocytic activity of leukocytes both before and subsequent to enzymatic therapy.

The studies to be described in this article may be divided into the following categories:

1) Effect of injections of SK–SD on the total number, supravital staining, and motility of white blood cells in pleural exudates of patients.

2) Differential counts of exudates following injections of SK–SD in patients.

3) *In vitro* studies of phagocytic activity of leukocytes following injection of SK–SD in patients.

4) Relationship of extracellular desoxyribose nucleoprotein to clumping of leukocytes in exudates.

5) Effect of SK–SD on the staining of cell nuclei of viable and degenerated cells in exudates as determined by the Feulgen reaction.

6) Correlation of enzymatic depolymerization as determined biochemically with cellular changes in pleural exudates of patients.

MATERIALS AND METHODS

I. *Streptococcal concentrate.* SK–SD [3] was contained in crude and partially purified concentrates prepared and measured by methods described by Christensen (4). In most instances the preparations contained both enzymes in high concentrations. Occasionally, a concentrate was used that had a very high SK titer and a very low SD titer. Although it would have been desirable to employ preparations containing SK or SD alone, at the time this work was in progress the only preparations available for therapeutic use contained both enzymes. Further studies with purified SK and SD, respectively, are being carried out. Special mention will be made of enzyme titers in individual protocols.

All enzyme preparations employed for studies *in vitro* were dialyzed against Simm's buffer [4] at 4° C for three days in order to attain a physiologically balanced solution with an osmotic pressure comparable to that of human serum. The osmotic pressures were then calculated from cryoscopic determinations. The freezing points had a range of − 0.55° to − 0.58° C. No preservative was used in these preparations. Sodium bicarbonate and phosphate buffers were employed exclusively in the initial preparation and purification of the enzymes.

The crude material contained about 16–20 units of SK (4) and 3–4 units of SD (4) per gamma of nitrogen and was found to be relatively stable in a concentrated solution in the cold. The protamine purified preparations contained about 150–200 units of SK, about 40–200 units of SD per gamma of nitrogen and were relatively unstable over long periods of time unless a non-specific protein was added as a protective colloid. In the case of the crude lyophilized enzyme, stability was attained by using a final concentration of about 2% of the crude enzyme, and in the case of the protamine purified, it was assured by adding human serum, that had been shown to have low anti-streptokinase and anti-streptodornase titers, to a final concentration of 0.2% to 2%.

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[1] This study was supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

[2] For brevity, Streptokinase is referred to as SK and streptococcal desoxyribonuclease as SD. SK–SD indicates the mixture.

[3] A number of the preparations were supplied by Lederle Laboratories.

[4] The formula of Simm's buffer is the same as Simm's Physiological Solution "X7" manufactured by Microbiological Associates.

All studies *in vitro* were performed with duplicate aliquots. The controls were set up with the same enzyme solutions which had been previously incubated at 56° C until enzyme activity was no longer demonstrable.

II. *Cell counts.* Total counts and white blood cell counts were done in duplicate by standard methods, except that thick, highly cellular exudates were diluted in saline to bring the cell numbers within the range of the methods. Dilution with saline also served to effect a partial depolymerization and dissolution of the highly viscous, extracellular desoxyribonucleic acid (DNA) usually present. The white cells tended to clump markedly in the presence of the latter, unless primary dilution was effected. Tenth normal HCl with added Loeffler's methylene blue was used as the final diluting fluid for white blood cell counts.

III. *Supravital staining and motility of leukocytes*

A. *Supravital stain.* Supravital staining was performed according to standard techniques (5, 6). National Aniline Neutral Red, certification No. NX14, was used in varying concentrations depending on the number of cells in the fluid studied. The cover-slip technic was used with silicone stop-cock grease (Dow Corning) instead of vaseline. Thick, highly cellular exudates were diluted with Simm's solution, 1:1 to 1:5, when necessary, to facilitate observation and promote an optimal concentration of cells for efficient, non-toxic staining. By varying the dilution of the cells and the concentration of neutral red, the proportion of stain to cell numbers was readily adjusted in spite of the wide variety of exudates tested.

Two hundred to 600 cells from the exudates of patients were observed in duplicate wet preparations on a warm stage (37.5° C) under oil immersion. A mechanical stage was utilized to count the cells in a standard Z pattern on the slide. The *per cent* staining supravitally was calculated, and the *number* staining supravitally was in turn calculated from the percentage value obtained above and the total white blood cell count. At least two observations were made within two hours, although many samples observed for considerably longer time intervals were found to check with the original data.

B. *Motility.* Enumeration of the cells exhibiting motility was performed at the same time and under the same conditions as was the enumeration of cells taking supravital stain. Motility was expressed as the per cent of those cells which took the supravital stain that showed definite pseudopods and/or pseudopod formation with protoplasmic streaming. The actual number of motile cells was calculated from the percentage value obtained above, and the number of white blood cells staining supravitally.

IV. *Phagocytosis.* Experiments on phagocytosis were carried out with a strain of rough pneumococcus, R 36N/C. The bacteria were washed three times in neopeptone broth and diluted in the broth to concentrations of 10^3 to 10^8 bacteria per milliliter. Nine-tenths ml. samples of exudate were added to 0.1 ml. of washed pneumococci in each of two test tubes and the tubes

were rotated at 15 r.p.m. (7, 8) in a waterbath at 37.5° C for 30 to 60 minutes, respectively. The mixture was then smeared on a slide, stained with Wright's stain, and 300–500 white blood cells were observed for ingested bacteria (9). The highest per cent of leukocytes showing phagocytosis was taken as the "phagocytic activity" of that exudate regardless of the concentration of the organisms. In most cases, maximum phagocytosis was attained at one hour at a concentration of 10^8 bacteria per milliliter. Although there was an excess of bacteria, the number of bacteria per leukocyte was not used as an index because the ratio of bacteria to viable leukocytes in the original mixture could not be rigidly controlled. In those experiments in which active streptococcal desoxyribonuclease was present, depolymerization of the DNA in the cell nuclei occurred after traumatic rupture of some of the cells during the process of smearing the exudate. This was prevented by the addition of sodium citrate to a final concentration of 4% just before smearing the slides.

V. *Desoxyribose nucleic acid (DNA)* [5]

A. *Feulgen stain.* Thick and thin smears of exudates were air dried and Feulgen stained as described in a previous report (2). No fixative was used. Fast Green FCF was used as a counterstain.

B. *Extraction.* The polymerized DNA in the sediment and cells was washed with cold, citrated saline, and extracted with 5% trichloracetic acid (10).

Analysis of the partially depolymerized DNA in the supernatants was far more difficult because of the presence of interfering substances such as hexoses and degradation products from protein hydrolysis following hot trichloracetic extraction. Preliminary results with a method of extraction utilizing crystalline pancreatic desoxyribonuclease [6] in 5% bovine albumin have shown that removal of interfering protein and extraction of DNA were relatively complete in this method. Treatment of the sample under standard conditions was followed by precipitation of the protein with 5% trichloracetic acid at room temperature. The acid soluble DNA in the filtrates was then measured by chemical methods.

C. *Analysis.* A modification (11) of Dische's diphenylamine reaction (12), utilizing the difference in absorption at 6,100 Å and 6,500 Å, was used for DNA analyses. Because protein may interfere in the reaction (13, 14) a modification of the cysteine method of Stumpf (15) was employed as a check. The modification used consisted of varying the amount of sample from 0.5 ml. to 1.5 ml., and the concentration of sulfuric acid from 70% to 90% (in order to maintain a final concentration of 63%). A uniform lot of "aged" sulfuric acid solu-

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[5] Inasmuch as DNA was the substance specifically tested for in exudates from patients, a sharp distinction will not be drawn between desoxyribose nucleoprotein and DNA; and desoxyribose nucleoprotein will usually be referred to as DNA.

[6] Obtained from the Worthington Laboratories, New Jersey.

tion was used and separate curves calibrated for each concentration.[7]

The temperature of the reaction was kept below 40° C. When 80% or 90% sulfuric acid was used, the reagents were iced and sulfuric acid was added to the samples very slowly in an ice bath. The analyses were performed in Beckman DU and Model B spectrophotometers using DNA standards of the sodium salt of thymonucleic acid prepared in this laboratory (N/P ratio 1.6). The modification described increased the sensitivity of the original method threefold.

VI. *Other biochemical determinations*

A. *Phosphorus.* Total acid soluble phosphorus, inorganic phosphorus, and organic acid soluble phosphorus were determined by methods previously described (2).

B. *Nitrogen.* Acid soluble nitrogen was determined on 8% trichloracetic acid filtrates by micro-Kjeldahl and Nesslerization.

VII. *Collection of samples of exudate from patients.* No anti-coagulant was used or found to be necessary in the purulent exudates. Heparin was used routinely in non-purulent exudates. Since high concentrations of heparin interfere with phagocytosis and motility (16), the concentration used did not exceed approximately 10 mgms. %. Moreover, at this concentration, little or no inhibition of SD occurs.

RESULTS

1) Effect of injections of SK–SD on the total number, supravital staining, and motility of white blood cells in pleural exudates of patients

The local pleural cellular response to injections of SK–SD has been observed in patients with *non-purulent, serous effusions* resulting from bronchogenic carcinoma, cardiovascular-renal disease, and tuberculous pleurisy.[8] Enzyme concentrates with combined unit dosages ranging from 20,000 SK–4,000 SD to 100,000 SK–25,000 SD contained in 5–10 ml. of diluent were employed and total WBC counts were made before and 24 hours after the injection.

The results may be summarized as follows: the average of the total number of WBC's before injection was 827 per cu. mm. Twenty-four hours after injection there were 11,380 WBC's

[7] For reasons that are not understood, full color development and uniform results were not obtained unless "aged" sulfuric acid solution and a standard time interval (60 seconds) for adding the acid to the sample were used. The sulfuric acid solution was heated to 56° C for 12 hours, then allowed to remain undisturbed for several months.

[8] These observations have been made over a period of several years by different observers.

per cu. mm. The smallest increase was from 1,650 to 2,750 cells per cu. mm.; the greatest was from 400 to 26,800 cells per cu. mm. The change was largely accounted for by an increase in polymorphonuclear leukocytes. When two succeeding injections were given to the same patient in this preliminary group within three to five days after the effects of the first had subsided, there was a six to eight times greater outpouring of cells following the second injection.

In two instances, enzymes inactivated by heating for 24 hours at 56° C were injected in patients with pleural effusions. In one case, the number of cells pre- and 24 hours post-treatment were 1,100 and remained unchanged. In the other, the number of cells pre- and 24 hours post-treatment were 75 and 180, respectively. It is of interest that a significant cellular increase occurred later in these patients when they were treated with an active enzyme preparation of the same lot as the inactivated material. These results were confirmed in two other instances, when the SK–SD was applied locally to a clean, granulating ulcer, and a special bandage utilized to pool the enzyme and collect the exudate. These limited observations suggest that the degree of local cellular reaction is specifically associated with activity of SK–SD. The increase did not seem to correlate well with enzyme dosage or pyrogenic reactions. Further studies of the several factors contained in the streptococcal concentrates that specifically influence the degree and type of reaction are in progress.

Eleven patients with *empyema with thick, purulent* exudations received 19 injections of SK–SD in varying amounts in the successful therapeutic range. There were four cases of pneumococcal empyema (two infected, two sterile), three tuberculous, one due to anaerobic streptococcus, one to *B. pyocyaneus,* and two sterile empyemas of undetermined etiology. The dosage of streptokinase per injection varied from 200,000 to 400,000 units, and that of streptodornase from 5,000 to 200,000 units. When a patient received more than one injection, the time interval between injections was determined by clinical indications and varied from two days to two weeks. Specimens were procured before injection and one hour or more post-injection during the first day and usually at 24 hour intervals thereafter for the duration of the

observations. Drainage of the pleural fluid was not effected until 24 hours after the injection. Appropriate biochemical and cytological studies showed that specific enzyme action had taken place in each case (1, 2).

The cytological effects of injections of SK–SD in these empyemata are shown in Figure 1.

From Figure 1 it may be noted that there was a wide range in the total number of WBC's in the pleural exudate before therapy, varying from 4,000 to 500,000 cells per cu. mm. Serial counts following the introduction of the enzymes indicated the initial response usually became evident

during the first hour post-treatment and reached its maximum at 24 hours. In two instances the count was lower after 24 hours (patients McM, first injection, and BAT, second injection. Although not recorded in Figure 1, the maximum number of WBC's was usually maintained for 24 hours and diminished during the ensuing week to or below the pre-injection level. Many factors pertaining to the underlying disease and degree of response to treatment controlled the number of cells present during the post-injection period. Under optimal clinical conditions, it was not unusual for the local WBC count to fall markedly
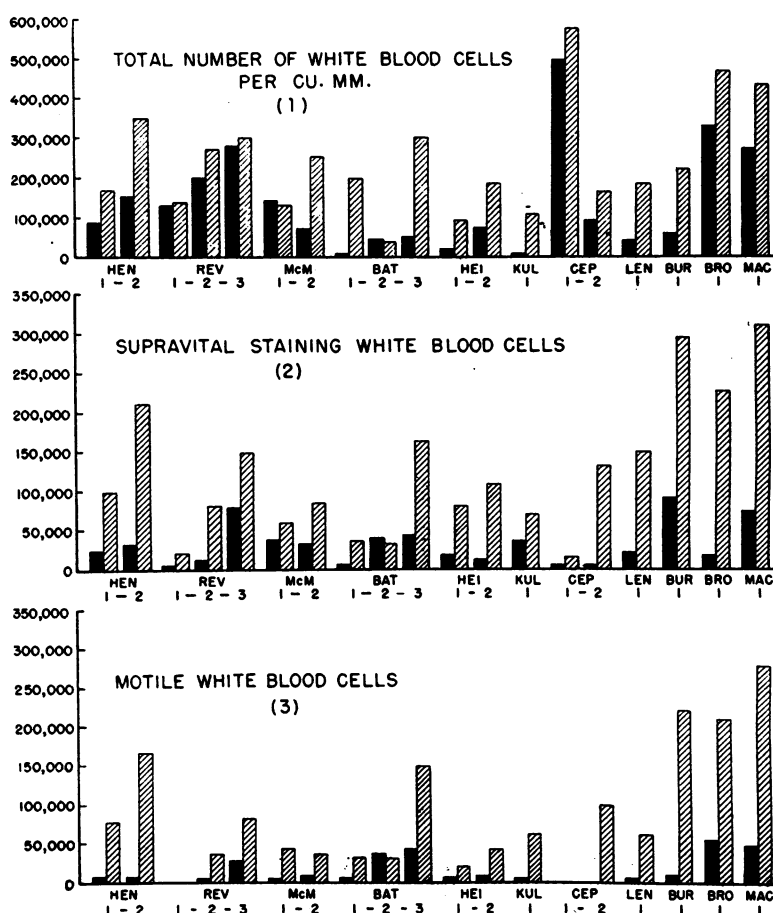

FIG. 1. EFFECT OF INJECTIONS OF SK–SD ON THE TOTAL NUMBER, SUPRAVITAL STAINING, AND MOTILITY OF WHITE BLOOD CELLS IN PLEURAL EXUDATES OF PATIENTS

The solid bar represents the number of white blood cells before injection and the cross-hatched bar, the number of white blood cells 24 hours after injection.

When a patient received more than one injection, this is denoted by the number under his initials.

in three to five days. A case demonstrating this point is described in a later section.

Even though the number of white blood cells per cu. mm. varied inversely with its dilution by pleural fluid, the increase in total number of WBC's occurred in spite of the increase in pleural fluid usually observed following the injection of the SK–SD.

It was not clearly established, however, whether the accumulating cells emerged from the site of the inflammation within the tissues or were released from deposits of exudate undergoing lysis by the enzymes. Since a considerable number of cells in the original, highly viscous exudate were dead or degenerated, the studies on vital staining strongly suggest that a major portion of the increase in white blood cells was due to an outpouring of fresh viable cells.

Associated with the change in the total number of white blood cells, the *number* of cells staining supravitally and those exhibiting motility increased in a significant and striking manner which was emphasized by the increase in *per cent* of cells taking supravital stain and exhibiting motility. These phenomena were consistently noted after each injection with one exception. For the 19 injections, the mean increase in white blood cells per injection per 24 hours was 112,000 with a standard deviation of 71,400, and the mean increase in motile white blood cells was 72,400 with a standard deviation of 67,900. *Thus, the increase in cells staining supravitally constituted 83% of the increase in total WBC's, and the increase in motile cells constituted 77% of the cells staining supravitally.*

Further analysis of the data in Figure 1 shows that the number of cells staining supravitally and exhibiting motility had undergone a much greater proportionate increase than the total white count. The ratio of the mean of the post-injection counts to the initial counts indicates that the total white blood cell counts increased about twofold, the supravital staining about fourfold, and the motility about sixfold in 24 hours. For example, in the cases of BRO, and MAC (Figure 1), the final or post-injection white count is less than 1.5 times the pre-injection white blood cell count whereas the cells staining supravitally and exhibiting motility increased after injection to more than four times the pre-injection level. In addition, when

the per cent of the total cells staining supravitally was noted for a number of consecutive time intervals following an injection, there was usually a 30–60% rise in supravital staining in 24 hours. If two or more injections were given to the same patient at one to two week intervals, the response following the second injection was about twice that following the first. It would seem, therefore, that the cytological formed elements in these purulent pleural fluids undergo a nearly complete "turnover" following injection of the SK–SD from a static state with a preponderance of dead, degenerated cells to a dynamic state with a majority of fresh, viable cells.

2) *Differential counts of exudates following injections of SK–SD in patients*

The differential counts of exudates from patients with a variety of diagnoses have had one common denominator following therapeutic use of the concentrate. No matter what the initial or
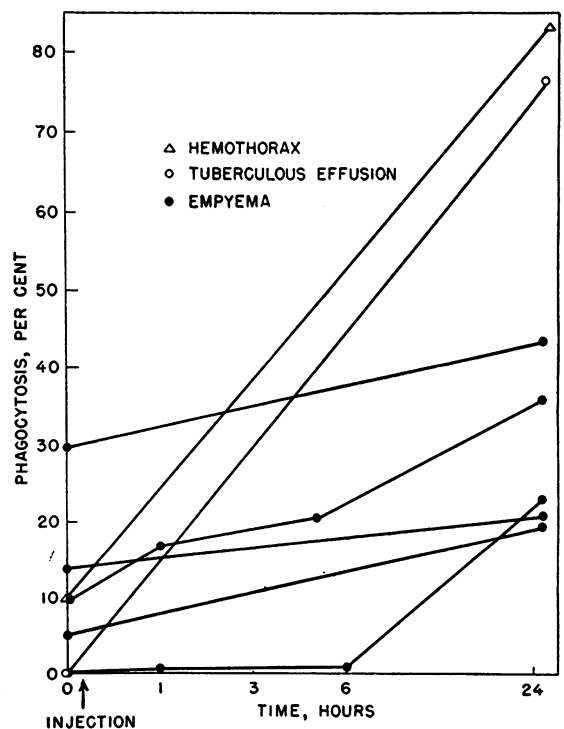

FIG. 2. IN VITRO PHAGOCYTIC ACTIVITY OF LEUKOCYTES IN PLEURAL EXUDATES REMOVED AT VARYING INTERVALS AFTER THE LOCAL INJECTION OF SK–SD IN PATIENTS

The ordinate represents the per cent of cells showing phagocytic activity.

final quantitation, regardless of the ratios of the initial cell types, 24 hours after the injection the cells were largely those of the polymorphonuclear leukocytic series. Where elimination of infection or marked improvement occurred following the injections, the polymorphonuclear series was replaced by mononuclear cells of various kinds, a few lymphocytes at first, then monocytes, macrophages and endothelial cells. It was not understood what part, if any, the macrophages played. They were rarely present until long after the acute reaction had subsided.

3) In vitro studies of phagocytic activity of leukocytes following injection of SK–SD in patients

Following the injection of SK–SD into the pleural cavities of patients, a significant rise in the proportion of leukocytes containing ingested R pneumococci was demonstrable in each of the seven cases presented in Figure 2, as contrasted with the pre-injection specimen. In the cases of frank empyema, three tuberculous, one pneumococcal, and one sterile empyema of undertermined etiology, the phagocytic activity 24 hours after first therapy was increased by 10–28%. It will be recalled that the per cent of cells staining supravitally in these cases was considerably higher. The other two cases, hydrothorax and hemothorax, respectively, showed a much higher elevation of the *per cent* of phagocytic activity in the presence of SK–SD, nearly comparable to that obtained *in vitro* in normal whole blood, with or without SK–SD. These findings, with the low or absent glucose levels in the chest fluids following injection, suggest that the phagocytic ability of the leukocyte may approach a maximum limited by its nutritional milieu when the exudative coating is thick and the total cell population relatively large (17, 18).

Since the purulent exudates from these patients with empyema usually contained 100,000–200,000 white blood cells per cu. mm. while the exudates from the cases of hydrothorax and hemothorax contained 1,000–10,000 WBC's per cu. mm., the total number of cells available for phagocytic activity was considerable in the cases of empyema.

4) Relationship of extracellular desoxyribonucleic acid to clumping of leukocytes in exudates

The white blood cells of purulent exudates, particularly in empyema of long standing, were clumped in masses of varying sizes which were broken up when SK–SD was introduced (1). Although several thousand slides were reviewed, gross clumping of the white blood cells in purulent chest fluids was not observed in the absence of extracellular DNA. DNA, rather than fibrin, was particularly significant in the clumping phenomenon, since the cellular aggregations were usually seen in cases of thick, purulent empyema, which usually had a high DNA and a low fibrinogen content. They have not been seen in effusions or hemothorax where both the extracellular DNA and the fibrinogen were low. The clumping of WBC's in chronic purulent infections was probably associated with the high viscosity of the extracellular DNA and was broken up by the streptodornase. This view was also supported by the failure of preparations containing high titers of streptokinase with very low streptodornase content to depolymerize the DNA or break up the clumps of white blood cells in purulent exudates.

5) Effect of SK–SD on the staining of the cell nuclei of viable and degenerated cells in exudates as determined by the Feulgen reaction

When fixed tissue, sections and smears were treated with pancreatic desoxyribonuclease (19, 20, 21), the nuclei of the treated cells no longer stained with the Feulgen stain even though the time of exposure was only five to 10 minutes. It is likely that the permeability of the cell was altered by the preliminary fixation, thus allowing the enxyme to depolymerize the DNA in the nuclei. In order to determine further the action of SK–SD on living leukocytes the enzyme concentrate was added to freshly drawn human blood in a test tube, the mixture incubated for several hours at 37.5° C and subsequently smeared without fixation. Most of the nuclei in the white blood cells were clearly delineated by the Feulgen stain. It was apparent from the morphological appearance of the few cells that did not stain with the Feulgen technique but stained readily with the counterstain, that they were ruptured in the process of smearing. When sodium citrate was added after incubation and just before smearing, all the cells stained without difficulty. This was probably a result of the binding of magnesium ion, an essential cofactor of desoxyribonuclease, in the

form of a poorly ionized citrate complex. By trial and error, it was found that "protection" of the smear with citrate was not necessary with therapeutic concentrations of streptodornase.

Since SD did not act upon the nuclei of fresh living cells but caused complete depolymerization of the DNA in fixed dead cells, its effect on exudates was studied where many stages of cell maturation and degeneration have been shown to co-exist.

The microscopic examination of Feulgen-stained smears of exudates (without fixation) disclosed two general morphological cell-nuclear states: (a) those whose nuclei appeared clear cut and sharply delineated with full nuclear staining and (b) those degenerating cells in which the cytoplasm appeared intact but karyorrhexis and/or karyolysis were taking place. The number of degenerating cells divided by the total number of cells counted, times 100, was defined as the "per cent degeneration." [9]

The per cent degeneration is reproducible to 1–2%: (a) when a suitable sampling technique is employed, (b) when slides are examined by the same microscopist, (c) when 300–500 cells are counted, and (d) when a monochromatic light source is utilized with Wratten gelatin light filters G–15 and B–58.

The per cent degeneration was calculated from those cells that were visible on the Feulgen stain. Since depolymerized DNA is not stained by the Feulgen method, "degenerated" cells that had undergone extensive depolymerization were lost to visibility by this method, although the "ghost forms" were visible under direct microscopy, and therefore included in the total cell counts done by the usual methods. For this reason, the sum of the per cent degeneration and the per cent of cells staining supravitally did not equal 100% unless the cell population was moderately fresh (24–48 hours).

When SK–SD was added to purulent exudates *in vitro* the enzyme-treated specimen exhibited a low per cent degeneration of WBC's as contrasted

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[9] The nuclei in the degenerating cells are undoubtedly undergoing depolymerization but by definition have already undergone further depolymerization than that which Pollister measured with methyl green stain (22).

TABLE I

Effect of SD on the per cent degeneration of cell nuclei in purulent pleural exudates in vitro

Time in hours	Per cent degeneration of control after incubation with heat-inactivated SD	Per cent degeneration after incubation with SD
0	52	2
15	76	6
35	97	6
60	99	0

with a high per cent degeneration in the controls. The extent of degeneration after enzyme exposure was invariably reduced to 10% or less of the total, whereas the initial per cent degeneration may have been 90% or more.

The data in Table I were obtained on sterile empyema fluid, incubated under sterile precautions at 37.5° C. At varying time intervals 5 ml. aliquots were removed and incubated with an excess of SK–SD in a phagocyte rotor for two hours at 37.5° C. Sodium citrate was added to both control and enzyme-treated specimens after incubation, smears made and Feulgen stained, and the per cent degeneration determined on each.

The striking decrease in the per cent of degenerating cells after incubation of the exudate with SK–SD was closely correlated with a comparable decrease in the DNA of the cellular sediment (Figure 3). Experiments illustrating this were set up as follows:

White blood cells were isolated from the peripheral blood of seven patients by a modification of the method of Osborne (23) and a sterile exudate was utilized from a patient with tuberculous effusion. No extracellular desoxyribose nucleoprotein was demonstrable in any of the samples by Feulgen staining. Consequently, any chemical evidence of DNA depolymerization (acid soluble DNA) was referable to the degenerated nuclei. The white cell preparation was incubated under sterile conditions at 37.5° C, aliquots subjected to an excess of SK–SD in a phagocyte rotor for two hours, supravital stains made, citrate added, smears made and Feulgen stained, and the per cent degeneration determined as in the previous experiment. The cellular sediments obtained by centrifugation were washed with citrated physiological saline and the DNA content determined by chemical analysis. The per cent decrease in DNA was calculated from the DNA in the sediment of the enzyme-treated and control aliquots.

The close correlation of cytochemical and chemical methods emphasizes the value of observing changes in the per cent of degenerating leukocytes by utilizing the Feulgen stain as a measure of SD activity. It may also be noted in Figure 3 that there is no evidence of enzymatic depolymerization of the DNA in the fresh non-degenerated cell by SK–SD. This finding is supported by evidence presented above that staining of the viable cell nucleus by the Feulgen reaction is unaffected by SK–SD in high titers.

Studies in which SK–SD was incubated *in vitro* with living leukocytes obtained from the peripheral blood and exudates have also failed to reveal any effect of the enzymes on the supravital
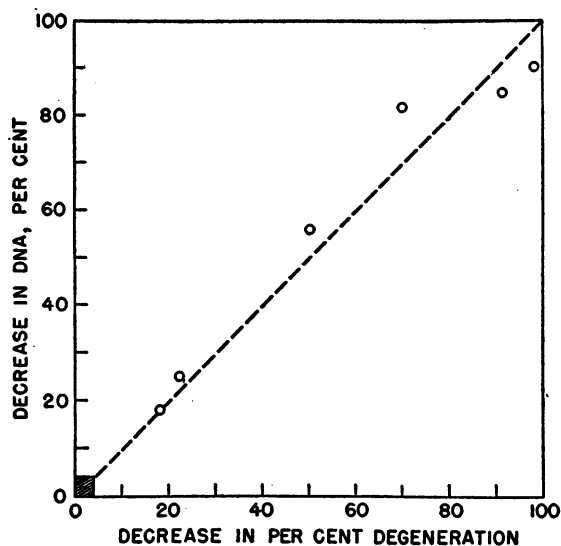
FIG. 3. RELATION OF DECREASE IN DNA AND PER CENT DEGENERATION OF CELL NUCLEI IN WHITE BLOOD CELLS AFTER TREATMENT WITH SK–SD

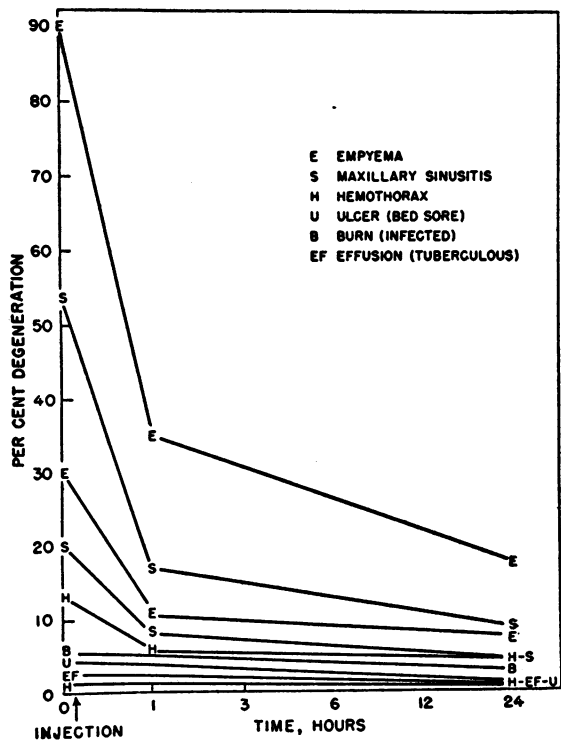The square represents five determinations.

FIG. 4. EFFECT OF SK–SD ON THE PER CENT DEGENERATION OF CELL NUCLEI IN LEUKOCYTES IN EXUDATES IN MAN

Representative data are shown from a variety of exudates.

staining and motility of the leukocytes, as well as on their ability to phagocyte the R pneumococci.

A decrease in degenerating cells has been repeatedly observed *in vivo* (Figure 4) as well as *in vitro* following administration of SK–SD.

The degree of change in per cent degeneration *in vitro* and *in vivo*, respectively, is primarily a function of the initial level, *i.e.*, the amount of available substrate (2); the higher the initial per cent degeneration, the greater the decrement following injection of SK–SD. For example, speci-
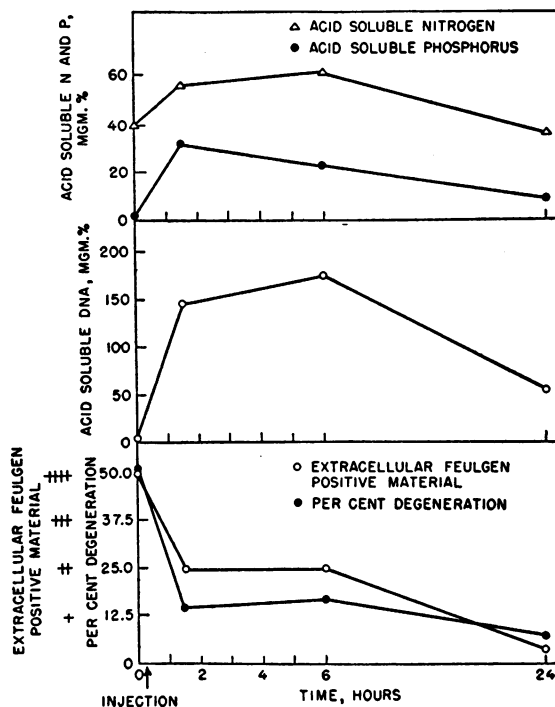

FIG. 5a. INJECTION OF SK–SD IN A PATIENT WITH THICK PURULENT EMPYEMA

Serial sampling was performed by thoracentesis.

mens of exudates derived from areas of chronic, stagnant suppuration that drained with difficulty, such as empyema or sinusitis, exhibited a high per cent of degeneration before therapy. On the other hand, the free flowing material obtained from purulent lesions that had continuous open drainage (bed sore, ulcer, infected burn) and from closed non-purulent lesions (hemothorax and tuberculous effusion) showed a relatively low initial per cent degeneration. However, regardless of the conditions of the diseased sites, or the initial level of the per cent degeneration, the per

cent of degenerating cells in the exudate 24 hours after enzymatic treatment was regularly reduced to 5–10%. This phenomenon has been observed in 65 purulent and non-purulent exudates from 33 patients with widely disparate diagnoses, and was indicative of the significance of SK–SD in eliminating degenerating cell forms.

It should be clear that the changes noted above were acute changes, the result of an immediate response to a short stimulus. When the exudative reaction had spent itself, the new cells, too, ultimately degenerated.

6) Correlation of enzymatic depolymerization as determined biochemically with cellular changes in pleural exudates of patients

In order to compare the effect of SD on the extracellular desoxyribose nucleoprotein with the cellular changes that occur concomitantly, Figure 5a and Figure 5b were prepared from simultaneous chemical and cellular data derived from two patients with thick, purulent empyema.

Immediately after the injection of SK–SD, there was a sharp fall in the extracellular Feulgen-positive material and the per cent degeneration, with a marked rise in the acid soluble desoxyribose nucleic acid, organic acid soluble phosphorus, and acid soluble nitrogen liberated into the supernatant of the exudate (Figures 5a and b).

The cytological changes occurred early and progressed to a maximum at about 24 hours, in contrast to the faster moving biochemical response. Thus, there was a moderate increase in total white blood cells, and a marked increase in motile, viable cells during and immediately following the period of greatest enzymatic depolymerization. The disproportionate increase in supravitally stained, motile cells may be visualized as closing the gap between an initial heterogeneous, non-viable, pathological cell population and the relatively homogeneous, viable cell population elicited by the acute enzyme action. Since this is accompanied by an absolute decrease in the number of cells that do not stain supravitally immediately follow-
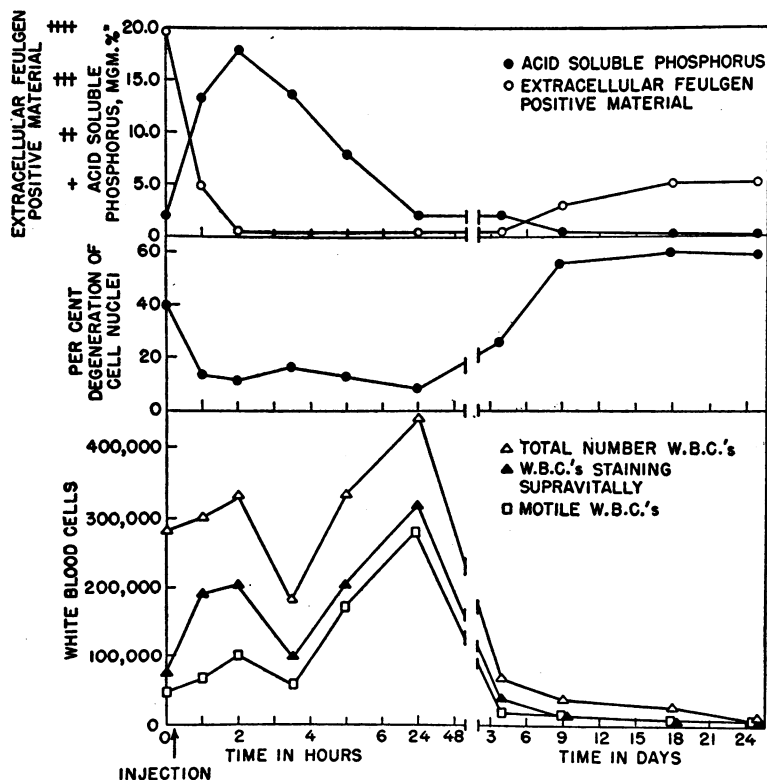

Fig. 5b. Injection of SK–SD in a Patient with Thick Purulent Empyema

Serial sampling was performed by thoracentesis.

ing the enzyme injection, it may be concluded that the marked increase in fresh, viable cells capable of phagocytosis was accompanied by a concomitant fall in the *actual number* of degenerated cells. It should be emphasized that the rapid changes just described did not occur in a large number of control thoracenteses. One interesting disparity has been observed in the cell counts of *in vitro* and *in vivo* experiments. Following incubation of exudates with SK–SD *in vitro,* the total cell numbers showed only a slight decrease when the counts were done by the usual methods even though Feulgen stained preparations showed a marked decrease in the per cent of degenerating cells; and as the degenerating cells decreased, due to enzyme action, the ghost forms increased. In the *in vivo* observations, on the other hand, the ghost forms or degenerated cells were markedly decreased. Although the phagocytosis of ghost cells or cell fragments by living leukocytes has not been directly observed, it seems likely that phagocytosis, proteolysis by leukoproteinases (24, 25, 26) and absorption (27) account for the obliteration of the cellular forms *in vivo,* and with respect to the effect of enzymatic debridement accounts for the local cleaning of tissues.

The patient's improvement following this total response may be noted by the abrupt fall in total cell count terminating the acute phase (Figure 5b) with subsequent convalescence of the area. In this instance also, phagocytosis by polymorphonuclear leukocytes and possibly macrophages was probably of importance in the progressive diminution of the total cell numbers. Simultaneous chemical and cytological studies of the acute phase have been repeatedly made with comparable results and the subsequent course of the disease in a patient with a bacteriologically controlled empyema usually followed the pattern outlined above.

DISCUSSION

From the findings recorded in this report it is apparent that a number of changes in the local cytological picture at the site of inflammatory exudative areas in patients, are rapidly evoked by the introduction of SK–SD. Some of these alterations are manifested by the appearance in the free fluid at the site of the disease, of a considerable percentage increase of living, active, motile leukocytes. From the point of view, therefore, of

cleaning the tissues at the site of disease, the effectiveness of the enzymes, acting as liquefying agents on their specific substrates, is supplemented by the appearance of cellular elements that probably contribute to debridement by phagocytosis.

It is of interest to note that a very considerable proportion of the total cell count of chronic inflammatory exudates may be composed of degenerating and degenerated cells. The usual methods for doing white blood counts give little indication of this fact. By a combination of white blood counts, supravital staining, and Feulgen staining, the differentiation of living, degenerating, and degenerated cells can be made.

Inasmuch as the supravital staining, motility, phagocytosis, and the degree of polymerization of the DNA in the nucleus of fresh viable cells do not seem to be affected by SK–SD, the cytological studies have failed to yield any evidence that the active enzyme systems affect living leukocytes. Either the surface of the viable cell is impermeable to SK–SD or, if streptodornase is absorbed the living nuclei inhibit depolymerization (28, 29).

SUMMARY

1. The introduction locally of streptococcal concentrates containing streptokinase-streptococcal desoxyribonuclease, into areas of inflammatory exudation in patients, was followed promptly by an outpouring of living polymorphonuclear leukocytes which usually reached its maximum 24 hours after the injection. In four instances in which both enzymes, inactivated by heat, were injected locally in patients, only minimal local cellular reaction was observed. Since the concentrates employed contained mixtures of streptokinase and streptococcal desoxyribonuclease, the extent to which each individually contributed to the cellular outpouring has not been determined.

2. Changes in the local cytological pattern caused by the injection of the enzymes were characterized by a marked decrease in degenerating and degenerated cellular forms, and a rapid rise in supravitally staining, motile, phagocyting leukocytes.

3. Depolymerization of desoxyribonucleic acid effected by treatment with streptococcal desoxyribonuclease caused a breaking up of clumped leukocytes, the conglomeration of which was ap-

parently due to a matrix of desoxyribose nucleo-protein.

4. Liquefaction of highly viscous purulent fluid by streptococcal desoxyribonuclease, as determined by preparations stained by the Feulgen method, resulted in the disappearance of extracellular desoxyribonucleic acid and the nuclei of degenerating cells. The nuclei, as well as other evidences of viability of living cells, remained unaffected. Biochemical evidence of depolymerization accompanied the cytological changes.

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