POLYMORPHONUCLEAR INVASION OF WOUNDED CORNEAS*

Inhibition by Topically Applied Sodium Salicylate and Soybean Trypsin Inhibitor

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During the first 3 days after injury, wound healing is usually considered to be passive in nature and to consist of the exudative and cleaning-up phases of phagocytosis. This is followed on the 3rd and 4th days by the first appearance of the new connective tissue. It seems reasonable to assume that changes determining the formation of this new connective tissue must be set into motion during these first 2 or 3 days. In order to understand the initiation and control of wound healing it is, therefore, most important to know the cause-and-effect relationships of the physiological and chemical events occurring in the first 3 days after injury.

The first detectable changes in the tissues following wounding are those of the inflammatory process. It is generally agreed that the stimulus which initiates inflammation is derived from the injured cells (1). Among the early events of the aseptic traumatic inflammatory process is the polymorphonuclear invasion of the wounded area. This phenomenon is believed to result from the liberation of chemotactic substances by the injured cells. The only chemotactic substances which have actually been obtained from injured tissues are polypeptides (2–8). To date no evidence has been found to show that the products of fat or carbohydrate breakdown are the active chemotactic factors in injured tissues for polymorphonuclear leukocytes (8). According to Spector peptides containing 8 to 14 amino acid residues, and derived from certain types of proteins, are active inflammatory agents (8). A source of such polypeptides would, of course, be proteolytic activity arising in the injured tissues.

The activation of proteolytic enzymes in tissues by various injurious agents has been demonstrated by several investigators. (See Beloff and Peters (9), Ungar (10–12), Herberts (13–14), Scroggie *et al.* (15), and Weimar (16).)

The studies reported in this paper deal with (*a*) the time sequence of events leading to polymorphonuclear invasion of the wounded rat cornea, and (*b*) inhibition of this reaction by topically applied sodium salicylate and soybean

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trypsin inhibitor. The cornea is particularly suitable for these studies inasmuch as it is avascular and relatively acellular. The invading cells must migrate from the limbal tissues and limbal blood vessels, and quantitative counts of the invading cells can easily be made.

Methods

Standard Techniques.—Male rats weighing 250 to 350 gm. were used. The rats were lightly anesthetized (about 2 minutes) with ether; and by means of a sterile Graefe knife, a cut approximately 2 mm. in length was made as near the *center* of the cornea as possible. After the indicated experimental period the rat was killed with ether and its eyes enucleated and fixed in formalin-alcohol (2 parts 36.3 per cent neutral formalin, 1 part 88 per cent alcohol) for not less than 3 and not more than 6 hours. A strip of the cornea, which included the wound, was so cut that the wound lay at right angles across the corneal strip. A 1 to 2 mm. flap of sclera was left attached to each end of the strip for convenience in handling. These sections were next soaked for 1 hour in a saturated aqueous solution of *n*-amyl alcohol to loosen the epithelium and endothelium (17). These were then easily scraped off with the blunt end of a single edged razor blade. It was necessary to remove the epithelium to see clearly with the microscope through all depths of the stroma of the stained preparations. Further steps for preliminary dehydration, clearing, and rehydration before staining were carried out as described by Buschke, Friedenwald, and Fleischmann (17). The strips were stained for approximately 1 minute in Orth's lithium-carmine (diluted one-half with a saturated solution of lithium carbonate), destained for about 10 minutes in acid-alcohol (1 part HCl, 99 parts 70 per cent alcohol), rinsed in distilled water, and dehydrated by running through 70 per cent, 95 per cent and 2 changes of absolute alcohol for 10 minutes each. For clearing they were treated for 2 minutes in oil of thyme, followed by 2 minutes in oil of *Origanum*, blotted, and mounted in Canada balsam for microscopic examination.

With the aid of an oil immersion objective (675×) the polymorphonuclear cells were counted from limbus to limbus through all depths of the corneal stroma. Because of its transitional structure, the field immediately adjacent to the limbus was never counted. In this paper the counts are expressed as the number of polymorphonuclear cells per oil immersion field through all depths of the stroma.

Group I. Unwounded Eyes and Untreated, Wounded Eyes.—A series of unwounded eyes were examined to determine the average number of polymorphonuclear cells present in the normal cornea. To establish the pattern of polymorphonuclear invasion of the wounded cornea, wounds of 4, 5, 6, 11, 24, and 36 hours, and 2, 3, and 6 days were studied.

Group II. Wounds Treated with Sodium Salicylate or with Soybean Trypsin Inhibitor.[1]—

A. Wounds treated with sodium salicylate: The drug was used at 0.1 M concentration dissolved in saline, and applied topically as drops. All solutions were adjusted to pH 7.3–7.5 (using a pH meter). The eyes were bathed for 2 minutes (timed) at the following time intervals established after a long series of preliminary experiments. The first 4 drops were given at 10-minute intervals, the next 3 at 15-minute intervals, the following drops, if any, at 30-minute intervals.

B. Wounds treated with soybean trypsin inhibitor: This substance was first used as a 1 per cent solution made up in saline and adjusted to pH 7.3–7.5 (using a pH meter). It was applied topically as drops for 2 minutes (timed) at 10-minute intervals. Inasmuch as the 1 per

[1] 5× recrystallized. Obtained from Nutritional Biochemicals Corp.

cent concentration was only partially effective, it was increased to 2 per cent and this was used throughout the experiments described in this paper.

RESULTS AND DISCUSSION

Group I. Unwounded Eyes and Untreated, Wounded Eyes.—A small number of polymorphonuclear cells were found in the unwounded rat cornea, the average being 3.97 cells per oil immersion field (through all depths of the stroma). The invasion of the incised cornea by polymorphonuclear leukocytes began during the 5th hour after wounding, as is shown in Table I. Preliminary studies indicate that the invasion increased until 24 to 36 hours after injury when the average was 45 to 55 cells per field, and returned to normal in about 6 days.

TABLE I

Average Number of Polymorphonuclear Cells in Normal and Wounded Rat Corneas at Various Time Intervals after Injury*

Wound period	No. of eyes	Cells per field	95 per cent confidence interval
No wound	18	3.97	5.00– 2.94
4 hours	8	4.63	6.05– 3.21
5 "	11	14.39	18.43–10.35
6 "	26	23.32	30.64–16.00
11 "	10	26.21	36.19–16.23

* Expressed as the average number of cells per oil immersion field through all depths of the stroma.

The average number of cells per oil immersion field 4 hours after wounding was 4.63 ± 0.60 (standard error of the mean); 5 hours after wounding, 14.39 ± 1.81; and 6 hours after wounding, 23.32 ± 3.55. (See Table I).

Group II. Wounds Treated with Sodium Salicylate or Soybean Trypsin Inhibitor.—On the basis of the observations summarized in Table I, 6 hours after wounding was chosen as the earliest time period that could accurately be studied for the inhibitory effect of various drugs on polymorphonuclear invasion. The working hypothesis for the following experiments is that the wounding process causes the activation of a proteolytic enzyme (or enzymes) which, by the digestive action, liberates chemotactic substances (probably polypeptides), thus leading to polymorphonuclear invasion.

A. Wounds Treated With Sodium Salicylate.—This drug was used because it has been demonstrated to be an inhibitor of both pepsin (18) and fibrinolysin (19), two very different kinds of proteases. Furthermore, Ungar has shown that in guinea pig liver slices and skin slices sodium salicylate inhibits the proteolysis caused by anaphylactic and anaphylactoid agents (12) and by burning (11).

The results obtained with the various treatments are shown in the diagrams in Charts 1 and 2.

Chart 1, Diagram I.—Salicylate was applied topically throughout the 6 hour period of the wound. Polymorphonuclear invasion was 91 per cent inhibited. An average of 5.7 cells per field was found, compared with 23.3 for untreated 6-hour wounds and 4.0 for unwounded eyes. (Tables I and II.) Analysis with the t test shows that there is no difference between

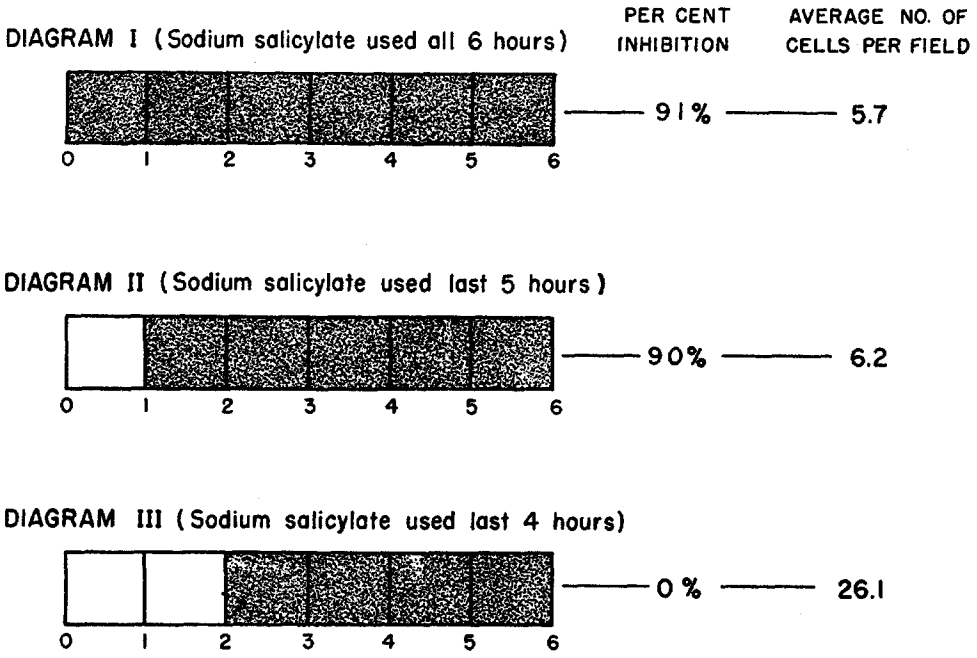

CHART 1. The influence of topically applied sodium salicylate on the polymorphonuclear infiltration of 6-hour corneal wounds. Each square in the diagram represents 1 hour of the 6-hour wound period. The shaded areas indicate the time periods during which the drug was applied.

the number of polymorphonuclear cells in unwounded eyes and in eyes with 6-hour wounds which have been treated for all 6 hours with salicylate ($P < 0.2 > 0.1$; Table II).

Chart 1, Diagram II.—Salicylate was next applied for only the last 5 hours of the 6 hour wound period. The polymorphonuclear invasion was again nearly completely inhibited (90 per cent). An average of 6.2 cells per field was found. (For the statistical analysis, see Table II, footnote ‡.)

Chart 1, Diagram III.—The wound was treated with salicylate for the final 4 hours only. There was no (0 per cent) inhibition of the polymorphonuclear invasion. An average of 26.1 cells per field was found. There is no difference between the number of polymorphonuclear cells found in these wounds, treated during the last 4 hours with salicylate, and 6-hour untreated wounds ($P < 0.8 > 0.7$; Table II).

In the experiment just described salicylate is used during the period preceding polymorphonuclear infiltration of the wounded tissue as well as during the period of cell migration. It is thus demonstrated that salicylate does not act directly on the polymorphonuclear cells to inhibit their migration. If chemotactic substances are involved in attracting the inflammatory cells to the site of injury, they would be expected to be active during the period pre-

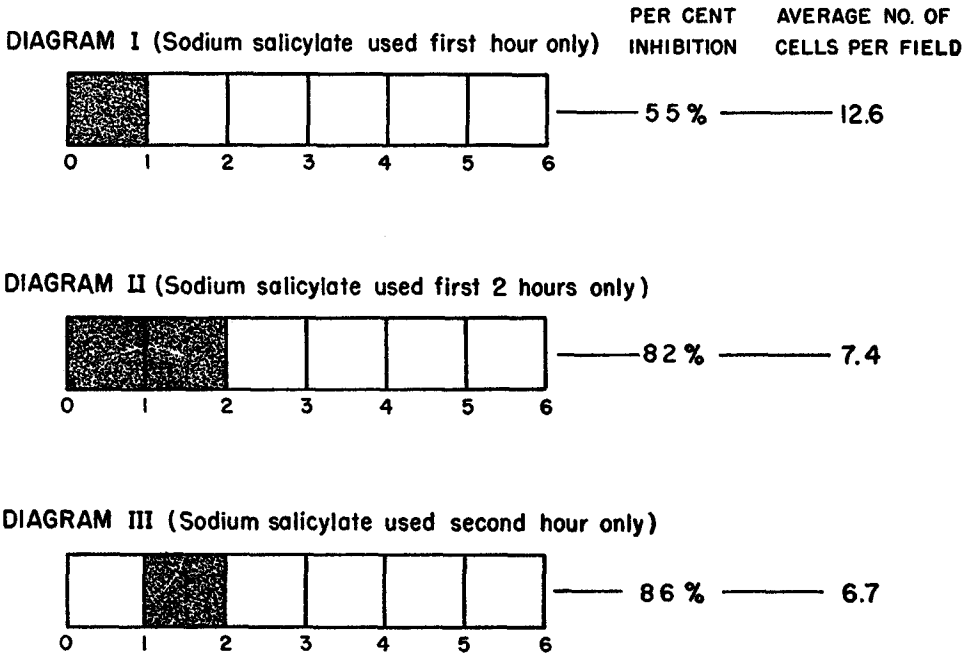

CHART 2. The influence of topically applied sodium salicylate on the polymorphonuclear infiltration of 6-hour corneal wounds. Each square in the diagram represents 1 hour of the 6 hour wound period. The shaded areas indicate the time periods during which the drug was applied.

ceding migration of the cells. It appears, therefore, that the salicylate does not produce its inhibitory effect by acting on chemotactic substances.

The results indicate that a reaction (or reactions) occurs during the 2nd hour of the wound which leads to polymorphonuclear invasion and that this reaction is inhibited by topically applied sodium salicylate. (See Chart 1, Diagrams II and III.) The following experiments were performed to test this hypothesis (Chart 2, Diagrams I, II, and III).

Chart 2, Diagram I.—The 6-hour wound was treated for the 1st hour only with salicylate. About 55 per cent inhibition of polymorphonuclear invasion was found. There was an aver-

age of 12.6 cells per field. Although some inhibition occurs, it is not enough to make these wounds significantly different from untreated 6-hour wounds ($P < 0.2 > 0.1$; Table II).

Chart 2, Diagram II.—When the wound was treated with salicylate for the first 2 hours only, the polymorphonuclear invasion was nearly completely inhibited (82 per cent). An average of 7.4 cells per field was found. The statistical analysis indicates that this treatment does not differ from 6-hour wounds treated continually with salicylate ($P < 0.4 > 0.3$; Table II).

Chart 2, Diagram III.—The 6-hour wound was treated with salicylate for the 2nd hour only. The polymorphonuclear invasion was again nearly completely inhibited (86 per cent). An average of 6.7 cells per field was found. Analysis with the t test indicates that the inhibition obtained by treating 6-hour wounds continually with salicylate may be accounted for by the 2nd hour of treatment ($P < 0.6 > 0.5$; Table II).

TABLE II

Influence of Topically Applied Sodium Salicylate on Polymorphonuclear Invasion of Wounded Rat Corneas

Period of treatment of 6-hr. wound	No. of eyes	Cells per field	95 per cent confidence interval	Probability that difference is due to chance*		
				Compared with no wound	6-hr. untreated wound	Compared with salicylate used all 6 hrs. of wound period
All 6 hrs.	9	5.67	8.69– 2.65	<0.2 >0.1	<0.01 >0.001	—
Last 5 "	4	6.20	11.07– 1.33	‡	‡	‡
Last 4 "	8	26.14	42.24–10.04	<0.001	<0.8 >0.7	<0.01 >0.001
First hr.	8	12.56	16.82– 8.30	<0.001	<0.2 >0.1	<0.01 >0.001
First 2 hrs.	8	7.35	9.76– 4.95	<0.01 >0.001	<0.05 >0.02	<0.4 >0.3
Second hr.	8	6.68	8.91– 4.45	<0.01 >0.001	<0.02 >0.01	<0.6 >0.5

* Analysis with Student's t test.

‡ For statistical analysis, refer to data for 2nd hour. Experiments testing the effect of sodium salicylate on the last 5 hours of the wound reaction were discontinued after treatment of the last 4 hours was found to be ineffective.

It was thus confirmed that a reaction (or reactions) occurring during the 2nd hour after wounding leads to polymorphonuclear invasion and that it is this reaction (or reactions) which is inhibited by topically applied sodium salicylate. The partial inhibition during the 1st hour shown in Chart 2, Diagram I is attributed to the continued presence in the 2nd hour of the salicylate applied during the 1st hour. If part of the reaction took place during the 1st hour, then the degree of inhibition found by treating the last 5 hours (Chart 1, Diagram II) or by treating the 2nd hour only (Chart 2, Diagram III) would not have occurred. The results shown in Chart 1, Diagram III (treating the last 4 hours) indicate that salicylate cannot stop the polymorphonuclear invasion once the reaction has occurred.

Preliminary experiments indicate that the inhibition by topically applied sodium salicylate is reversible and that about 60 per cent recovery occurs 24 hours after wounding. Saline alone, applied during the first 2 hours of the 6-hour wound, is without effect on the polymorphonuclear invasion.

B. Wounds Treated with Soybean Trypsin Inhibitor.—In this series of experiments soybean trypsin inhibitor was used to determine whether the reaction (or reactions) occurring in rat corneas during the 2nd postoperative hour and leading to polymorphonuclear infiltration is that of a protease. Both soybean trypsin inhibitor and sodium salicylate suppress fibrinolysin (15, 19), both prevent the proteolysis in tissue slices produced by burning

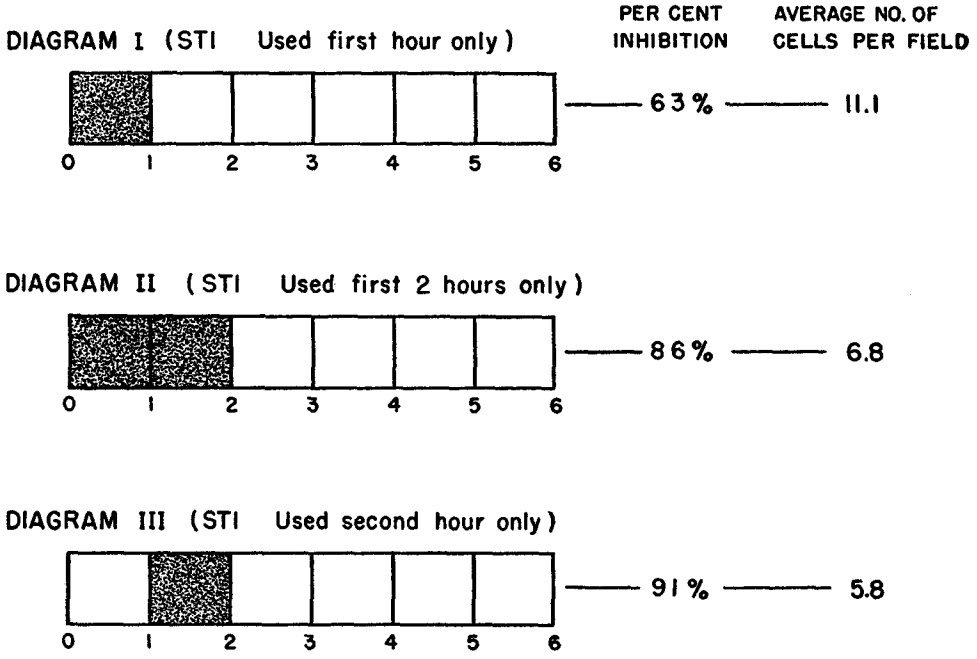

CHART 3. The influence of topically applied soybean trypsin inhibitor on the polymorphonuclear infiltration of 6-hour corneal wounds. Each square in the diagram represents 1 hour of the 6-hour wound period. The shaded areas indicate the time periods during which the drug was applied. STI, soybean trypsin inhibitor.

(11) or by anaphylaxis and anaphylactoid agents (12). Soybean trypsin inhibitor does not inhibit pepsin (20) in contrast to the effect of sodium salicylate (18).

The results obtained with soybean trypsin inhibitor are shown in Chart 3, Diagrams I to III, and Chart 4, Diagrams I to V.

Chart 3, Diagram I.—When the soybean trypsin inhibitor was applied for only the 1st hour of the 6-hour wound period, about 63 per cent inhibition of the polymorphonuclear invasion occurred. Under similar conditions salicylate caused 55 per cent inhibition. For wounds treated with soybean trypsin inhibitor the average number of polymorphonuclear cells per

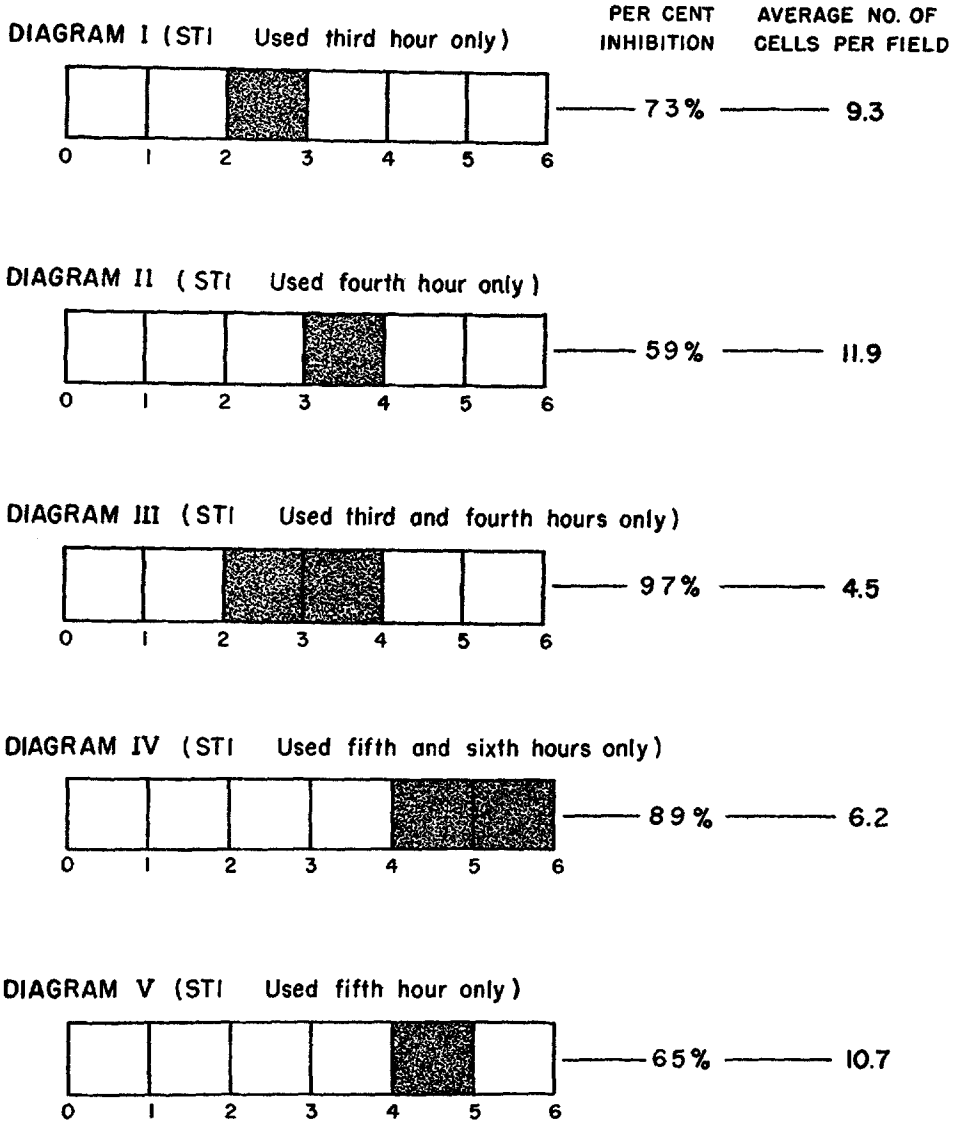

CHART 4. The influence of topically applied soybean trypsin inhibitor on the polymorphonuclear infiltration of 6-hour corneal wounds. Each square in the diagram represents 1 hour of the 6-hour wound period. The shaded areas indicate the time periods during which the drug was applied. STI, soybean trypsin inhibitor.

field was 11.1, for salicylate-treated eyes, 12.6. Although some inhibition was produced by this treatment, it was not enough to make these wounds significantly different from untreated 6-hour wounds ($P < 0.1 > 0.05$; Table III).

Chart 3, Diagram II.—If the wound was treated with soybean trypsin inhibitor for the first 2 hours, nearly complete inhibition (86 per cent) of the polymorphonuclear invasion occurred. An average of 6.8 cells per field was found, compared with 7.4 cells per field for salicylate-treated eyes. For the statistical analysis, see Table III, footnote (‡).

Chart 3, Diagram III.—If the wound was treated with soybean trypsin inhibitor for the 2nd hour only, 91 per cent inhibition of the polymorphonuclear invasion occurred. An average of 5.8 cells per field was found, compared with 6.7 cells for salicylate-treated eyes. There is no significant difference between the number of polymorphonuclear cells found in 6-hour

TABLE III

Influence of Topically Applied Soybean Trypsin Inhibitor on Polymorphonuclear Invasion of Wounded Rat Corneas

Period of treatment of 6-hr. wound	No. of eyes	Cells per field	95 per cent confidence interval	Probability that difference is due to chance*		
				Compared with no wound	Compared with 6-hr. untreated wound	Compared with 5-hr. untreated wound
First hr.	8	11.08	14.87–7.29	<0.001	<0.1 >0.05	—
First 2 hrs.	4	6.80	10.66–2.94	‡	‡	—
Second hr.	8	5.79	9.42–2.16	<0.2 >0.1	<0.02 >0.01	—
Third "	8	9.29	12.84–5.74	<0.001	<0.05 >0.02	—
Fourth "	8	11.85	17.53–6.17	<0.001	<0.1 >0.05	—
Third and fourth hrs.	7	4.49	6.13–2.85	<0.6 >0.5	<0.02 >0.01	—
Fifth hr.	8	10.69	15.31–6.07	<0.001	<0.1 >0.05	<0.2 >0.1
Fifth and sixth hrs.	8	6.23	9.19–3.27	<0.1 >0.05	<0.02 >0.01	—

* Analysis with Student's *t* test.

‡ For statistical analysis, refer to data for 2nd hour. Experiments testing the effect of soybean trypsin inhibitor on the first 2 hours of the wound reaction were discontinued after treatment of the 2nd hour only was found to be as effective.

wounds treated in this way and the number found in normal, unwounded eyes ($P < 0.2 > 0.1$; Table III).

The inhibition of polymorphonuclear invasion by treatment of the 6-hour wound during the first 2 hours with either soybean trypsin inhibitor or sodium salicylate was found to be virtually identical. The results with soybean trypsin inhibitor indicate that protease activity occurs during the 2nd hour after wounding and leads to polymorphonuclear invasion during the 5th and 6th hours of the wound reaction. This evidence, together with the knowledge that sodium salicylate inhibits some proteases, suggests that the sodium salicylate inhibits polymorphonuclear invasion of the wound by inhibiting a proteolytic enzyme (or enzymes). However, some other reaction (or reactions) occurring during this time period, either leading to the activation of

the protease(s) or activated by such a protease(s), may be the reaction which salicylate inhibits. Although the two agents behave similarly during the first 2 hours of the wound reaction, the soybean trypsin inhibitor differs entirely from the salicylate in its action on the last 4 hours of the 6-hour wound period. Whereas salicylate is completely ineffective in preventing the polymorphonuclear invasion when applied after the 2nd hour, soybean trypsin inhibitor is effective to varying degrees whenever it is applied during the last 4 hours.

Chart 4, Diagram I.—If the wound was treated for the 3rd hour only with soybean trypsin inhibitor, 73 per cent inhibition of polymorphonuclear invasion occurred. An average of 9.3 polymorphonuclear cells per field was found. The statistical analysis shows that there are more cells per field than in an unwounded eye ($P < 0.001$) but fewer cells per field than in an untreated 6-hour wound ($P < 0.05 > 0.02$). (See Table III.)

Since treating the 2nd hour only is nearly completely inhibitory, it does not seem likely that the proteolytic activity of the 3rd hour represents the same reaction as that of the 2nd hour. It seems more probable that a second reaction, perhaps extending into the 4th hour, actually takes place.

The two following experiments have been performed to test this hypothesis.

Chart 4, Diagram II.—Soybean trypsin inhibitor was applied during the 4th postoperative hour; 59 per cent inhibition was produced. An average of 11.9 polymorphonuclear cells per field was found. Although some inhibition is produced, it is not enough to make these wounds significantly different from untreated 6-hour wounds ($P < 0.1 > 0.05$; Table III).

Chart 4, Diagram III.—When the wound was treated during both the 3rd and 4th hours with soybean trypsin inhibitor, 97 per cent inhibition occurred. This agrees with the writer's tentative hypothesis that the 3rd and 4th hours represent a different phase of proteolytic activity from that of the 2nd hour. Analysis with the *t* test shows that the number of polymorphonuclear cells found in these wounds does not differ from the number found in unwounded eyes ($P < 0.6 > 0.5$; Table II).

Polymorphonuclear invasion has been shown to begin during the 5th postoperative hour (Table I). Therefore, the next series of experiments was performed to determine whether soybean trypsin inhibitor is also effective when it is applied during the time period when cell infiltration of the wounded area occurs.

Chart 4, Diagram IV.—The drug was applied during the 5th and 6th postoperative hours. Polymorphonuclear invasion was found to be nearly completely inhibited (89 per cent). Analysis with the *t* test indicates that the number of polymorphonuclear cells found in wounds treated in this way does not differ from the number found in unwounded eyes ($P < 0.1 > 0.05$; Table III).

Chart 4, Diagram V.—When only the 5th hour of the 6-hour wound period is treated, significant polymorphonuclear invasion occurs (compared with normal, unwounded eyes ($P < 0.001$; Table III). Since, according to the results shown in Table I and in Chart 4, Diagram IV, polymorphonuclear infiltration would not have started before the beginning of the treatment, the cells must have migrated into the wound area after the treatment stopped. With the onset of cellular invasion thus delayed approximately 1 hour, these wounds would be

expected to be similar to 5-hour untreated wounds. The statistical analysis shows this to be so ($P < 0.2 > 0.1$; Table III).

Polymorphonuclear leukocytes possess proteolytic activity (21). It is possible that the soybean trypsin inhibitor suppresses these proteases to prevent the cell migration in a way as yet unknown. This inhibition might occur because reversible softening by proteolysis of the cement substance of the capillaries is prevented so that diapedesis cannot occur. Proteolytic enzymes are known to digest the cement substance (22, 23). Perhaps ameboid movement is paralyzed by inhibition of the proteolytic enzymes of the cells. It may be simply a case of negative chemotaxis (2).

From these experiments it appears that a proteolytic enzyme (or enzymes) is activated during the wound reaction and that this proteolytic activity leads to polymorphonuclear invasion of the wounded area. It would seem that there may be at least two different stages of proteolytic activity involved in the time period preceding polymorphonuclear invasion: (*a*) the 2nd hour of the wound reaction, inhibited by both salicylate and soybean trypsin inhibitor, and (*b*) the 3rd and 4th hours after injury, suppressed by soybean trypsin inhibitor only. The inhibitory effects of salicylate and soybean trypsin inhibitor occurring during the 2nd hour may be on the same reaction (or reactions) or these drugs may suppress responses which are closely related steps leading to polymorphonuclear invasion.

The nature and number of proteolytic enzymes involved in the events leading to polymorphonuclear invasion of the wounded rat cornea can be assessed only by enzyme determinations of the wounded tissues at various time intervals after injury. Such studies are now being undertaken in this laboratory. Investigations are also being carried out to determine whether substances extracted from the wounded tissues at various time intervals will overcome the inhibitions produced by salicylate and by soybean trypsin inhibitor.

SUMMARY

Polymorphonuclear invasion of the wounded rat cornea is quantitatively described. The inflammatory cells enter the wounded tissue during the 5th postoperative hour. They steadily increase in number until they reach a maximum between 24 and 36 hours and return to normal by about the 6th day.

Six hour wounds are used to evaluate the influence of topically applied drugs on the polymorphonuclear infiltration of the wounded area. Sodium salicylate is an effective inhibitor of polymorphonuclear invasion when it is used during the 2nd postoperative hour, but it is without effect when it is applied after this time. Soybean trypsin inhibitor prevents the invasion of polymorphonuclear cells when it is administered during the 2nd hour only, or the 3rd and 4th hours together, or the 5th and 6th hours together.

The results indicate that activation of a proteolytic enzyme (or enzymes) in the injured corneal tissue is an essential step leading to polymorphonuclear invasion.

During the first 2 postoperative hours the anti-inflammatory effects of both drugs are nearly identical and may reflect an inhibition of the same proteolytic reaction or some different but closely related reactions.

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