Growth-promoting Factor in Exudates, Mechanism of Repair, and Preneoplastic-like Responses*

VALYMENKIN

(Department of Experimental Pathology and the Agnes Barr Chase Foundation for Cancer Research, Temple University School of Medicine, Philadelphia, Pa.)

Berenblum, in surveying the role of irritation in carcinogenesis, distinguishes nonspecific hyperplasia from a specific, preneoplastic type of hyperplasia (1). He rejects the idea that hyperplasia per se can bring about the formation of a tumor (2). Rusch and Kline have suggested the activation of dormant tumor cells by noncarcinogenic stimuli such as certain tissue injuries (16).

Some investigators question whether chronic cystic mastitis is to be considered a true precancerous lesion. Others, including Ewing, Warren, Bungeler, and Kuru, are of the opinion that this condition may in some cases predispose to the development of malignancy (3–5, 17). The earlier studies of the writer have indicated that severely injured cells at the site of inflammation liberate various factors capable of explaining the biological manifestations of inflammation (11, 13). One of the factors present in exudates displays growth-promoting properties, and a possible role in the mechanism of repair has, therefore, been ascribed to it (6, 11). This proliferative factor originally recovered in the exudates of rabbits is absent in blood serum, and the repeated injections of the irritant utilized, namely, croton oil, when emulsified with blood serum, have failed to induce any growth-promoting effects (6, 11).

The observations of the present communication concern themselves with the hyperplastic effects of the growth-promoting factor, when administered over a prolonged period, on both breast tissue and cutaneous epithelial structures. The induced hyperplastic condition resembles in many cases the picture encountered in the chronic cystic mastitis of human beings. The recovery of a growth-promoting factor in exudates is probably of significance in explaining the mechanism of repair in inflammation. Its ability to induce upon repeated injections a condition resembling that seen in chronic cystic mastitis as well as marked cutaneous epithelial hyperplasia may render such a factor a useful tool in the further studies of the relation of long-standing inflammatory reactions to the production of neoplasia.

METHODS AND MATERIALS

The proliferative factor was obtained from canine exudates following the injection under pentobarbital anesthesia of 1.5 ml. of turpentine into the right pleural cavity of dogs. In a few animals a different irritant was utilized. This consisted of an emulsion of 0.5 ml. of 5 per cent croton oil in olive oil. The exudative material was withdrawn at varying intervals ranging from 1 to 4 days following the injection of the irritant. The exudate was dialyzed against 8 times its volume of distilled water, while the cellophane tube was kept under constant agitation by attachment to either an electric or an air mixer. Dialysis was thus continued for a period of about 6 hours. Upon cessation of this procedure, the diffusate was collected and concentrated to one-tenth of its volume by evaporation in vacuo at about 40° C. in a water bath. When not in use the concentrated diffusate was stored in a refrigerator. All the glassware had been previously sterilized in a dry oven. In only some instances were penicillin G potassium and dehydrostreptomycin (60 μg. and 100 μg/ml, respectively) added to the concentrated diffusate.

The concentrated diffusate was purified further and then tested in several experiments. This consisted in treating the diffusate with acetone in concentrations of 1:1. A precipitate formed which gradually became heavier by maintenance of the acetone-diffusate preparation for 24 hours in a refrigerator. The supernatant was mostly decanted and the sediment centrifuged at 4000 r.p.m. for 20 minutes. The precipitate was washed twice, each time with small quantities of distilled water followed by brief centrifugation at about 3000 r.p.m. The

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washed acetone precipitate was suspended in physiological saline (0.9 per cent NaCl) and kept in a refrigerator when not in use.

The injections of the concentrated diffusate or of the acetone precipitate of the diffusate were performed about 5 times/week in doses of 0.5 ml. into or in the vicinity of the nipples of nonpregnant rabbits. For control purposes, either the concentrated diffusate of canine blood serum or physiological saline was employed. Usually an untreated nipple was also studied for comparative evaluation of the effect by the diffusible component on other nipples. The period of injections varied, ranging from about 2 weeks to 10 or 11 months. Earlier studies published elsewhere (6) have indicated that repeated injections of an irritant in the form of croton oil emulsified with blood serum fail to induce any growth-promoting activity. For this reason, this additional control was not utilized in the present series of experiments.

When the animals either succumbed or were sacrificed, the nipples with the adjacent cutaneous tissue as well as the underlying mammary tissue were carefully removed, examined, and fixed in 10 per cent formaldehyde for subsequent microscopic examination. The total number of all rabbits studied in the various experiments was 94. Microscopic preparations were obtained by sectioning at three different levels of a given paraffin block. In doubtful cases, serial sections were performed. In this way tangentially cut sections were eliminated.

RESULTS

The procedure of dissociating the injury factor, necrosin, located in the indiffusible fraction of the dialyzed exudate from the proliferative factor in the diffusate has been described and illustrated elsewhere (9, 11, 13). It was clearly demonstrated that the concentrated diffusate of a canine exudate had practically no injurious effect on the rabbit skin, in contrast to the effect of the euglobulin, necrosin, in turn extracted from the indiffusible fraction (9, 11). Furthermore, as already pointed out, the growth effects were not ascribable to the influence of a foreign protein or one of its derivatives, as indicated by the fact that canine blood serum diffusate induced no appreciable proliferation on rabbit tissue.

A series of nonpregnant rabbits injected repeatedly with the concentrated diffusate of exudate into or in the vicinity of the nipples yielded varying degrees of hyperplasia of the breast tissue. Hyperplastic reaction was gauged by the dilation and increased number of conspicuous ducts or acini, and also by the character of their lining epithelium. The density of the stromal connective tissue served to some extent as a rough measure of the amount of newly deposited connective tissue. The results of repeated injection of the concentrated diffusate of exudate on 77 rabbits are summarized in Table 1. The number of injections varied from 11 to 126, and the course of administration ranged between 2 weeks and 11 months. As described elsewhere (11), the effect on the breast tissue proper, within a period of 1 month, revealed hyperplasia of that tissue with considerable dilation of the ducts and proliferation of the lining epithelium. The resulting picture was found to be somewhat reminiscent of that encountered in human cases of chronic cystic mastitis. Connective tissue deposition in the interductal spaces was at times present to a striking extent. In one instance, newly formed fibrous tissue induced, within 3 months, a picture in the breast reminiscent of an intracanalicular fibroadenoma (Fig. 1).

When the course of injections was lengthened for several months, besides cystic dilation of breast tissue, atypical proliferation of the duct epithelium became a conspicuous microscopic feature (Fig. 2). Grossly, the breast tissue appeared as irregular and enlarged vesicular structures underlying the nipple itself. The marked proliferation of the lining epithelium at times occupied a considerable portion of the lumen. The basement membrane appeared occasionally to be poorly defined, and the proliferative epithelial cells seemed to be gradually infiltrating into the stroma. The absence of similar hyperplastic responses was found in the untreated nipple of the same animals. Repeated local injections of the acetone precipitate of the concentrated diffusate likewise induced cystic dilation and proliferation of the lining epithelium (14).

The utilization of several nipples in one and the same rabbit, and at the same time the retention of an untreated nipple as control, would rule out the possibility that the growth effects observed in injected nipples are referable to the known spontaneous cyclical proliferative changes which occur at times in nonpregnant rabbits. The untreated breast tissue failed to show any comparable effects, such as illustrated in Figure 2.

Another convenient site in which to study the effect of the diffusible proliferative factor of inflammatory exudates is the follicular and cutaneous epithelium in the vicinity of the nipple. Whereas in the skin area around an untreated...
nipple the hair follicles and the skin epithelium
displayed nothing remarkable (Fig. 3), repeated
injections of concentrated diffusate material for
6–7 months yielded marked hyperplastic responses
of the epithelium, accompanied by varying
degrees of keratosis and even dyskeratosis (Fig. 4,
Table 1). The basement membrane was poorly
defined, and there was a suggestion of invasiveness
of irregularly arranged epithelial cells. Mi-
totic figures were likewise found. The picture some-
what resembled that encountered in human cases
of pseudoepitheliomatous hyperplasia (12, 14).
The repeated injections of physiological saline for
as long as 6 months failed to induce any hyper-
plastic response (Fig. 5).

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>PROLIFERATIVE EFFECT FOLLOWING REPEATED INJECTIONS OF CONCENTRATED DIFFUSATE OF INFLAMMATORY EXUDATES</td>
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<tr>
<td><strong>NO. RABBITS AND COURSE OF ADMINISTRATION OF DIFFUSATE</strong></td>
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<tr>
<td>Total no. of animals injected with material in or around nipples: 77</td>
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<tr>
<td>Range of duration of injections: 2 weeks to 11 months</td>
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<tr>
<td>Range in the no. of injections: 11–196</td>
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<td><strong>TYPE OF EFFECT PRODUCED ON:</strong></td>
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<td><strong>BREAST TISSUE</strong></td>
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<td>Within 1 month (11–27 injections):</td>
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<td>Cystic dilatation of ducts; hyperplasia of lining epithelium; connective tissue proliferation in interductal space; at times infiltration of inflammatory cells (primarily lymphoid and macrophages). (See illustrations in reference 11.)</td>
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<td>Within 5 months (34–67 injections):</td>
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<td>Marked cystic dilatation of ducts; prominent hyperplasia of lining epithelium with at times squamous metaplasia and pseudostratification; occasional invasiveness into lumen of ducts by lining epithelial cells; basement membrane may be obliterated with suggestion of invasiveness; connective tissue proliferation in the interductal spaces (Figs. 1 and 2).</td>
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<td>Within 7 months (85–92 injections):</td>
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<td>Marked hyperplasia of epithelium around hair follicles; keratosis and dyskeratosis; disorganized epithelial proliferation (Figs. 4 and 6).</td>
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<tr>
<td><strong>CUTANEOUS EPITHELIAL STRUCTURES</strong></td>
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<td><strong>PRODUCTS OF DIFFUSATE</strong></td>
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| **Factor of exudates, and the absence of any similar corresponding effects following the repeated injections of blood serum diffusate, appear not to be caused by some differences in nonspecific factors such as pH or osmotic pressure. The pH of the concentrated exudate diffusate and that of the concentrated blood serum diffusate averaged about 8.4. There was no appreciable difference in the hydrogen ion concentration of the two types of diffusate. Osmotic pressure measurements by the depression in freezing point method likewise indicated, with both types of diffusate, an identical osmotic pressure, namely, 12.04 atmospheres.**

The studies with the injections of exudate and serum diffusate have included, to date, a study of 94 rabbits with the involvement of 193 nipples. Many of these animals are still alive and are being
studied further. Consequently, in a number of instances no histological studies have as yet been made. Nevertheless, in a considerable number of cases thorough histological examination of the breast tissue has been made. The results obtained are summarized in Table 2. In a group of 31 nipples repeatedly injected with the concentrated diffusate of exudate, 27 or 87.1 per cent have manifested evidence of hyperplasia. Note, in contrast, that only one out of nine nipples, or 11.1 per cent, injected with the diffusate of blood serum has revealed any trace of hyperplasia.

Finally, one may raise the objection that repeated injections of material in one nipple might per se be the cause of the proliferative effects. The absence of any such effects in the case of repeated injections of either concentrated blood serum diffusate or saline would indicate that repeated traumatic punctures are not the causative factor.

**TABLE 2**

<table>
<thead>
<tr>
<th>No. rabbits</th>
<th>Material injected</th>
<th>No. nipples injected</th>
<th>Nipples with hyperplasia*</th>
<th>Per cent with evidence of hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>Concentrated diffusate of exudate</td>
<td>100</td>
<td>27/31</td>
<td>87.1</td>
</tr>
<tr>
<td>17</td>
<td>Concentrated diffusate of blood serum</td>
<td>33</td>
<td>1/9</td>
<td>11.1</td>
</tr>
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</table>

* Ratio of nipples showing histological evidence of hyperplasia of breast tissue and/or of cutaneous epithelium to number of nipples studied.

Earlier studies had already pointed out that repeated injury by a strong irritant, e.g., croton oil emulsified with blood serum, was incapable of inducing any of the proliferative effects caused by the injections of exudative material (6).

Furthermore, to ascertain whether some of the effects obtained may not be referable to some turpentine, croton oil, or one of their derivatives in the diffusate, a series of experiments has been conducted by preparing homogenates of either canine liver, spleen, or rabbit liver, spleen, and kidney. These homogenates were dialyzed in the refrigerator for approximately 1 week against distilled water containing antibiotics (60 µg penicillin G potassium/ml and 100 µg dehydrostreptomycin/ml). The diffusates thus obtained were repeatedly injected into nipples of nonpregnant rabbits. One nipple was repeatedly treated with only the aqueous solution containing the antibiotics, whereas another nipple was kept as an untreated control. The results of such observations are illustrated in Figures 7 and 8. The diffusate of the homogenates of liver, spleen, or kidney induced, after repeated injections into the region of breast tissue of rabbits, cystic dilation of the ducts and hyperplasia of the follicular epithelium. This type of control shows that the results obtained with the concentrated diffusate of exudates are apparently not caused by contaminating traces of the irritant, such as croton oil or turpentine. Furthermore, the effects with the diffusate of the homogenates, and therefore mechanically injured cells of organs, would suggest that the growth-promoting factor of exudates is also liberated by injured cells.

The various chemical mediators present in inflammatory exudates, and described in earlier publications by the writer, are probably liberated by injured cells at the site of inflammation (11, 19). One of the main contentions for this interpretation is that a number of these mediators have been extracted from the homogenates of various tissues, such as liver, spleen, muscle, and kidney. This condition has been shown to be true in the case of leukotaxine, the leukocytosis-promoting factor of exudates (LPF), and of necrosin (7, 8, 11). In brief, the earlier studies of the writer have indicated that the exudate represents largely the products of cell injury and that the biologically active factors can in turn be recovered from such fluid. Furthermore, severe traumatic injury to cells during homogenization yields active factors with similar biological properties. Blood serum, on the other hand, fails to yield any of these factors. In this way it has been shown that a complete reproduction of the inflammatory process is evidently unnecessary for the recovery of the chemical mediators involved in inflammation. All that appears to be required is severe cellular injury, irrespective of whether this is brought about by inflammation or severe traumatization of tissues in vitro. In a similar manner, the present studies indicate that a growth factor, also diffusible, can be obtained from the homogenates of liver, spleen, and kidney. The results produced by the diffusible component of organs studied are quite similar to the proliferative effects elicited by the diffusible factor of exudates (cf. Figs. 7 and 8). The similarity of the biological effects, as well as the earlier studies with other mediators, is suggestive, even though the precise chemical identification of the factors in exudates and in the homogenates of organs has not as yet been established. The identity of biological effects induced by both diffusates of injured cellular material appears to be of definite significance in the interpretation of the results. Furthermore, the view
that the products of homogenization and those obtained by the development of inflammation are not of the same order may perhaps be true for some intracellular components; but, as pointed out above, this apparently is not true for the chemical mediators recovered in exudates. Finally, the writer in a recent study (15) has shown that at least some of the mediators of inflammation are associated with the very structural components released by homogenization of inflamed tissue or even by differential centrifugation of exudates. For instance, leukotaxine has been recovered in the mitochondrial fraction, and the leukocytosis-promoting factor has been identified in the soluble phase (S1 supernatant fraction). These newly obtained facts are not wholly surprising when it is recalled that inflammation is a manifestation of severe cellular injury and that this is likewise true of homogenized tissue. The actual morphological differences and the methods of inducing cellular injury appear to be of little significance as far as the release of the chemical mediators in an area of injury is concerned.

In conclusion, the foregoing observations point to the presence of a diffusible growth-promoting factor in pleural inflammatory exudates. This factor, upon repeated injections in the vicinity of the breast tissue of nonpregnant rabbits, induced a picture characterized by cystic dilation of the ducts and acini, hyperplasia of the epithelial lining, with occasional squamous metaplasia of these cells (Fig. 2), and varying degrees of fibrosis in the stroma (Fig. 1). The resulting lesions in some cases resembled those seen in chronic cystic mastitis as encountered in human beings (Fig. 2). Furthermore, the repeated administrations of the factor over prolonged intervals induced marked hyperplasia of cutaneous and follicular epithelium (Figs. 4 and 6). Since this factor had definite growth-promoting properties, its presence in exudates offers a reasonable explanation for the mechanism of repair in inflammation. None of the lesions has led to the formation of cancer, and therefore the term “pre-cancerous” for any of the induced lesions is unjustifiable. Nevertheless, the occasional squamous metaplasia observed in the breast lesions (cf. Fig. 2) and the production, with the factor, of a condition simulating in many instances chronic cystic mastitis—which is considered by some investigators to be a causative factor in the development of cancer—are to be noted.

The nature of the active principle responsible for the proliferative effects induced by the injections of the diffusate of exudate is not as yet clear. Only studies on the completely purified factor will allow definite inferences. This is at present under investigation. Spectrophotometric studies fail to indicate that it is in any way related to a nucleic acid derivative. Paper chromatographic studies, still preliminary in nature, carried out by my former associate, Dr. W. Kalnins, on the hydrolysate of the diffusate indicate that a peptide may be involved. At least seven or eight amino acid spots have been identified by him, and these are diagrammatically illustrated (cf. Chart 1).

The obvious question raised is whether a prolonged course of injections of the concentrated dialysate affects also any of the visceral organs. It was noted that many months following the repeated administration of the proliferative factor, the spleen contained large deposits of amyloid. This material not only appeared to be amyloid by the ordinary stains (hematoxylin and eosin), but likewise by using special stains, e.g., Congo red (Fig. 9). The amyloid was distributed at the periphery of the malpighian corpuscles. Amyloid deposits also appeared to be present in the liver. It was also present in the glomeruli of the kidney. It is therefore conceivable that amyloid may yet prove to be the result of a diffusible factor liberated by injured cells at the site of a long-standing inflammation. This point will be explored at

CHART 1.—Identification of amino acids on chromatogram of concentrated diffusate of exudate (identified by Dr. W. Kalnins).
greater length and will form the subject of a separate communication.

DISCUSSION

The observations presented in this communication indicate that injured cells at the site of inflammation liberate a diffusible component, which, upon repeated local injections, induces hyperplastic changes in the breast tissue of nonpregnant rabbits. These alterations appear in the form of cystic dilation of the ducts with varying degrees of proliferative activity on the part of the epithelial lining of these ducts. There may likewise be an abundant deposition of dense connective tissue in the stroma. The cutaneous and follicular epithelium adjacent to the injected areas around the nipples displays evidence of marked hyperplasia, keratosis, and dyskeratosis. Following several months of injections, the epithelial proliferating cells may at times show suggestive signs of invasiveness. Some of the lesions can perhaps be considered to be precancerous-like in appearance, although the establishment of autonomous growth, as yet an unproved point, would considerably strengthen such an interpretation. Furthermore, none of the induced lesions has developed into a cancer. The growth-promoting effects have not been obtained by utilizing as control the diffusate of canine blood serum.

The presence of a growth-promoting factor in inflammatory exudates which manifests marked proliferative effects can explain the mechanism of repair in inflammation (14). Its recovery, as in the case of the previously described mediators (11, 13), serves as further evidence that the biological manifestations of inflammation and repair can be referred to the liberation of various biochemical factors by the injured cell in an inflamed area.

The continuous exposure of some tissue to the proliferative factor described in this communication yields marked hyperplastic responses. The effects studied have occurred in the breast tissue of nonpregnant rabbits and also in cutaneous structures, including the hair follicles. In an earlier report similar effects on cartilagenous structures and skin have been described in rabbits injected repeatedly with the proliferative factor of exudate (6, 11). The factor can be readily dissociated by dialysis from the injury-inducing factor in exudates, termed necrosin. It is recovered in the diffusate, and the concentration and injection of this diffusate induces no appreciable degree of gross injury, but only proliferative or growth effects. The suggestive invasive tendency in some instances and the marked epithelial hyperplasia induced render it possible that we have in the proliferative factor of exudates a new tool to explore the relation of inflammation to the production of neoplasia. There are, as mentioned in the introduction, some investigators, notably Berenblum, who believe that nonspecific hyperplasia per se fails to induce ultimately a neoplasm (1). There are others who are of the opinion that the production of a neoplasm is a developmental process, passing through various stages of hyperplasia. The writer has in the past expressed the view that the diversity of methods which can induce a neoplasm suggests that a common denominator—in the form of a proliferative factor—may be liberated by cells which, in turn, may have been mildly injured by a variety of means, such as by chronic inflammation, a virus, or hormonal imbalance (6, 11). The presence of such a growth-promoting factor in the presence of a carcinogen, as will be pointed out in another paper, and possibly in combination with an inherent genetic factor, may perhaps favor the development of a neoplasm. It is conceivable that the growth-promoting or proliferative factor described may prove to be involved in this process. In brief, the presence of this endogenous growth factor liberated by injured cells may perhaps throw additional light on the role of a long-standing inflammatory process to the development of a neoplasm.


Fig. 1 (Rabbit 1).—Breast tissue following 34 local injections of concentrated diffusate for a period of 3 months. Note the dense deposition of connective tissue in the stroma producing the appearance of an intracanalicular fibroadenoma. ×255.

Fig. 2 (Rabbit 2).—47 injections of concentrated diffusate of exudate in the vicinity of the nipple over a period of 5½ months induced hyperplasia and squamous metaplasia of the lining epithelium in an acinus. This type of picture is consistent with that seen in the chronic cystic mastitis of human beings. ×255.
Fig. 3 (Rabbit 3).—Appearance of hair follicles in the immediate vicinity of an untreated nipple. There is no evidence of any hyperplastic response. This is the usual appearance of the normal hair follicles in the rabbit. Normal cutaneous epithelium fails to display such keratinizing pattern in untreated nipples. ×175.

Fig. 4 (Rabbit 5).—Marked hyperplasia around hair follicles within 7 months following 92 injections of the concentrated diffusate of exudate. Hyperplasia and keratosis are the striking effects of these injections when administered for months. ×175.
Fig. 5 (Rabbit 4).—Appearance of hair follicles and cutaneous epithelium in region of a nipple 6 months following 74 injections of physiological saline into that area. Note the absence of hyperplasia and keratosis. ×175.

Fig. 6 (Rabbit 3).—Appearance of hair follicles 6½ months following 85 injections in the nipple of the acetone precipitate of the diffusate of exudates suspended in saline. The hyperplasia and keratosis are striking but with no signs of any invasiveness. ×197.
FIG. 7 (Rabbit 6).—The effect on the follicular epithelium of 45 local injections into the nipple region of the diffusate of liver tissue homogenate as described in Fig. 8. Note the marked epithelial hyperplasia. The growth-promoting effect is similar to that obtained following injections of the diffusate of exudate (cf. Fig. 4). This evidence would suggest that the effect observed with exudates is probably owing to the liberation of a growth-promoting factor by injured cells at the site of inflammation. ×175.

FIG. 8 (Rabbit 6).—45 local injections of the diffusate of the homogenate of canine liver tissue into the nipple region of a nonpregnant rabbit induced cystic dilatation of the ducts with some epithelial proliferation of the lining epithelium. Part of a distended duct is shown. The period of injections lasted 4 months, and this section was made 11 months after cessation of injections. Such evidence strongly suggests that the growth-promoting factor is liberated by cells injured during the procedure of homogenizing the hepatic tissue. ×175.

FIG. 9 (Rabbit 7).—Spleen with considerable amyloid deposits following 101 injections, in or about the breast tissues, of the concentrated diffusate of exudates and also of the concentrated diffusate of blood serum. The period of injections extended for 9 months. ×135.
SUMMARY

There was, in the pleural inflammatory exudates studied, a growth-promoting factor. Its presence can offer a reasonable explanation for the mechanism of repair in inflammation. This proliferative factor was a diffusible component, and it could be readily dissociated by dialysis from the indiffusible injury factor in exudate, termed necrosin. There was evidence that the factor is liberated by injured cells, as indicated by its recovery in the homogenates of various tissues. The active principle could be purified further by precipitating it from the concentrated diffusate with acetone. Its exact chemical characterization awaits further studies.

In the breast tissue, the growth-promoting effects of the factor occurred in the form of newly formed acini, marked distention of the ducts, proliferation of the lining epithelium, and at times abundant deposition of connective tissue in the interductal stroma. The final picture was often similar to that encountered in the chronic cystic mastitis of human beings. The cutaneous and hair follicular epithelium, repeatedly exposed to the factor, likewise manifested marked hyperplastic responses.

ACKNOWLEDGMENTS

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Valy Menkin

Cancer Res 1957;17:963-969.