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Further Studies on Growth-promoting Factor of Exudates.* (25820)

VALY MENKIN

Henry Phipps Inst., University of Pennsylvania, and Dept. of Pathology, University of Kansas City School of Dentistry, Kansas City, Mo.

Earlier studies indicated that in inflammatory exudates there is present a diffusible growth-promoting factor capable of explaining reasonably the mechanism of repair following an inflammation(1,2). Repeated injections of this factor into breast tissue of non-pregnant rabbits induce lesions closely resembling chronic cystic mastitis(1,2). The cutaneous epithelial structures adjacent to treated nipples likewise display features of marked hyperplasia(2). Furthermore, evidence reported indicated that combination of growth factor of exudates and a carcinogenic hydrocarbon, methylcholanthrene, is followed by further enhancement of proliferative lesions(3,4). The concept has been developed that long standing inflammation may act as a cocarcinogen in presence of a carcinogen, be it exogenous or endogenous(4). The mechanism of action in this important role of inflammation in relation to development of neoplasia appears referable to liberation of growth-promoting factor of exudates(4). Evidence is here presented that activity of the factor appears destroyed by tryptic or ribonuclease digestion. Some of its biological properties are described.

Methods. The growth-promoting factor is present in concentrated diffusate of exudate and is obtained as described earlier(1,2). Chromatographic studies on acid hydrolyzate of the diffusate of exudates indicated association of a peptide with the active principle (2). Spectrophotometric absorption studies have also been conducted with a DU Beckman apparatus. With progress of inflammatory reaction from alkalinity to acidity(5), the diffusate assumes approximately the same pH as the exudate from which it has been obtained. The concentrated diffusate of alkaline exudates is adjusted with N acetic acid to approximately pH 4.5. A small precipitate forms, which augments further after refrigeration. It is then collected by centrifugation at 21,600 x g. This precipitate, washed twice with distilled water, is repeatedly injected into nipples of non-pregnant rabbits by technic previously described(1,2). Untreated nipples as well as nipples repeatedly injected with a buffer at pH 6.2 were likewise studied; the latter to control for the active growth factor, when injected in a medium at acid pH. Irreversibility of effects induced by growth-promoting factor has been tested by periodic observations of nipples for months following cessation of administration of the active principle. Chromatographic studies have been undertaken for presence of nucleotide structures in growth-promoting factor. The material is first hydrolyzed with 70% perchloric acid(6). Descending chromatography is uti-

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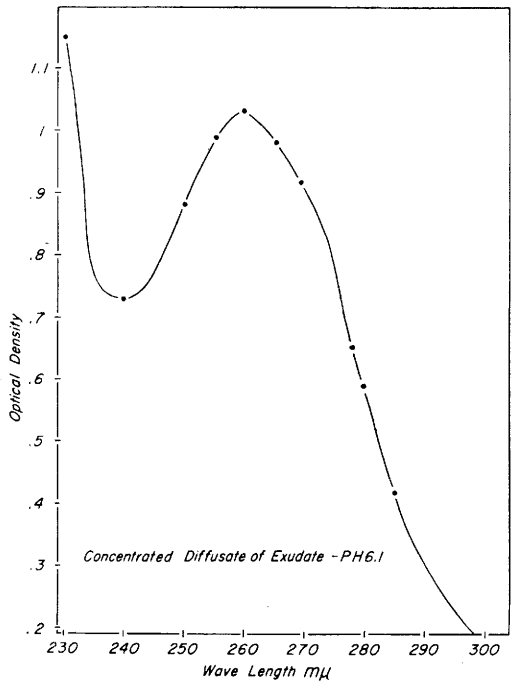

FIG. 1a. Ultra-violet absorption curve from concentrated diffusate of an acid exudate.

lized with 3 types of solvents as follows: 1) N-butanol, ethanol, 5 N HCl, (3:2:2), 2) Aqueous N-butanol (86% by volume), and 3) Isopropyl alcohol 170 ml, conc. HCl 41 ml, H_2O 39 ml(7). The purines, pyrimidines, nucleosides, and nucleotides present are eventually identified on dried Whatman No. 1 paper strips by an ultra-violet lamp. Enzymatic studies with the tryptic digest of growth-promoting factor of exudates have likewise been undertaken to determine whether biological activity is altered. The digest, brought to a boil, to inactivate crystalline trypsin is repeatedly injected into breast tissue of non-pregnant rabbits, and its effect compared on the same animal with that caused by the untreated growth-promoting factor. Ribonuclease hydrolysis of growth-promoting factor has also been undertaken to determine whether nucleotide groups play a part in its activity. These enzymatic studies are properly controlled by simultaneous repeated injections in other nipples of the enzyme *per se*.† In some experiments, to avoid injections with ribonuclease, the hydrolyzed ribonuclease growth factor preparation has been dialyzed overnight at 9°C against distilled water.

Results. I. Chemical groups concerned with activity of growth-promoting factor of exudates. The concentrated diffusate derived from acid exudate displays absorption curve with a peak at 260-265 mμ (Fig. 1a). This is consistent with presence of a nucleotide structure in association with the active biological fraction of growth-promoting factor.

The concentrated diffusate of an alkaline exudate is at alkaline pH. The absorption curve tends to flatten between 250 and 280 mμ. The difference between absorption curve of an acid diffusate from an alkaline diffusate is probably referable to a pH effect. As pointed out above, adjusting an alkaline diffusate to acid pH yields a small precipitate,

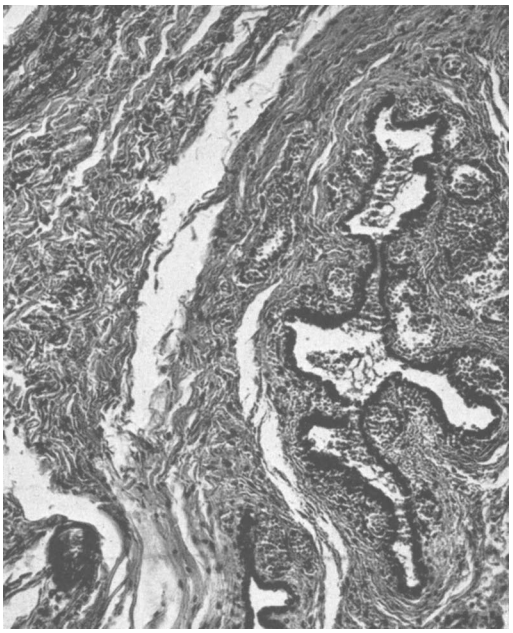

FIG. 1. Rabbit 52-94C. Microscopic picture of breast tissue in 3 wk following 7 injections of concentrated diffusate from canine exudate but previously incubated 1 hr with ribonuclease. Note that ducts and cutaneous epithelium in upper left corner reveal no striking dilatation or hyperplastic changes. Ribonuclease has evidently inactivated the active principle in growth-promoting factor of exudates. × 123.

† There is no evidence that ribonuclease utilized contains proteolytic activity. It is a preparation by Nutritional Biochemicals Corp., Cleveland, O.

TABLE I. Effect of Ribonuclease on Inactivation of Growth-Promoting Factor of Exudates.

Group	No. of inj. of growth-promoting factor with and without exposure to ribonuclease	Duration of exp. (mo)	Vol of breast tissue underlying nipple (cm^3)				
				Nipple inj. with			
			Untreated nipple	Conc. diffusate	Conc. diffusate but hydrolyzed previously with ribonuclease	Distilled water	Ribonuclease
I	24	2	.99	1.45	1.14	.87	
Ribonuclease-diffusate dialyzed prior to inj.				3.46	.89		
	28	2.5	.58	2.24	.16	.39	
				3.02	1.90		
	25	2.5	.23	.55	.12	.23	
				1.71	.10		
	15	1	.91	1.53	.48		
				.96	.81		
Avg	23	2	.68	1.87	.70	.50	
II	31*	3	.30	2.0	1.16	.51	
Ribonuclease-diffusate dialyzed for most but not all inj.				2.92	1.37		
	17*	1.5		1.53	.85	.23†	
				2.12	.65		
	11*	1	1.16	5.31	2.57	1.42	
			1.25	11.21	6.17		
Avg	20	1.83	.90	4.18	2.13	.72	
III	25	2	.10	1.56	1.33		.59
Ribonuclease-diffusate preparation not dialyzed prior to inj.					.64		.62
	26	2	.24	2.09	1.96		.35
					1.34		.31
	17	1.3	1.80	4.21	3.71	1.46	1.09
				5.18			
	24	2	.20	1.21	.35	.36	.11
				1.24			
Avg	23	1.83	.59	2.58	1.56	.91	.51

* 6 to 8 injections of ribonuclease-treated diffusate were performed without dialyzing hydrolyzed preparation.

† Received only 9 injections of distilled water.

which in an aqueous medium displays an absorption peak at 260-270 mμ.

Chromatographic studies with 3 different solvents indicate presence of nucleic acid derivatives. Both pyrimidine bases uracil, or its nucleotide, uridylic acid, and cytosine with its derivatives, were most often encountered. Adenine and guanine were also found with all solvents utilized. In brief, the chromatographic data confirm ultra-violet absorption studies, and indicate that a nucleotide appears associated with growth-promoting factor of exudates. Furthermore, ribonuclease tends either to inactivate or reduce the biological activity of the purified growth-promoting factor of exudates (Fig. 1). The results of observations on rabbits on effect of ribonuclease are given in Table I. Observations with ribonuclease hydrolysis were conducted, as a rule, on the same batch of diffusate material as injected in control nipples (Column 5, Table I). This fact rules out the possibility that results obtained are referable to diffusates chemically different in regard, for instance, to phosphorus or nitrogen contents.

Earlier chromatographic studies demonstrated presence of peptides in association with growth-promoting factor of exudates(2). The Biuret reaction on the diffusate of exudates is, as a rule, negative while the Ninhydrin reaction is positive. The test for pres-

ence of SH compounds with Nitroprusside is positive on concentrated diffusate of exudates.

Enzymatic digestion of the factor with trypsin inactivates its biological activity. Six rabbits repeatedly injected for many months into nipple region with tryptic digest of the factor display absence of proliferative effect by either gross or microscopic examination.

In conclusion, inactivation of the growth-promoting factor with either ribonuclease or trypsin suggests that both nucleotide and peptide structures may play an important role in ultimate chemical configuration of growth-promoting factor of exudates.‡

The possibility that a pteridine may also be involved is not consistent with data obtained by spectrophotometry. Pteridines besides having a peak at about 270 mμ reveal a subsequent peak between 320-420 mμ(8). Such a secondary peak was not encountered in measurements conducted on growth-promoting factor of exudates.

II. A. *Irreversible induced effects by proliferative factor.* When administration in breast tissue of growth factor in concentrated diffusate is shortened to about 2 months, and consists of 20 to 25 local injections, upon cessation of injections, the enlarged nipple tends to regress to normal size. When, however, injections are markedly increased and maintained for longer interval, in spite of cessation of injections, a state of irreversibility develops and volume of breast tissue not only fails to regress but it may also progressively increase. The animal shown in Fig. 2 and 3 had received 61 injections of concentrated diffusate of exudates for almost 5 months. Some nipples received also supplementary paintings with 1% methylcholanthrene. The animal was followed for 6 months following cessation of administration of the factor. As seen by comparing nipples B of Fig. 2 and 3, 3 months after discontinuing injections of

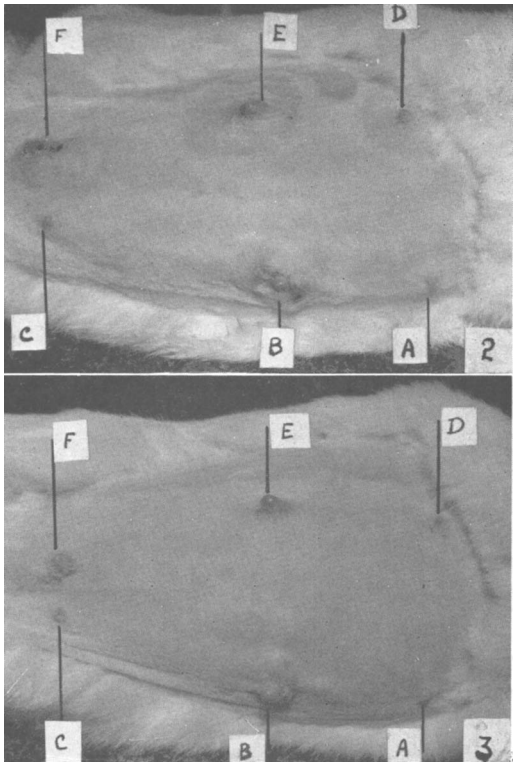

FIG. 2. Rabbit 27-07. For almost 5 mo nipples of this non-pregnant rabbit were treated as follows: A—untreated; B—61 injections of concentrated diffusate of exudate; C—61 injections of physiological saline; D—61 paintings with 1% methylcholanthrene in acetone (first 22 paintings consisted of methylcholanthrene in olive oil, 5% and 1%); E— and F—61 injections of concentrated diffusate of exudate plus 61 paintings with 1% methylcholanthrene in acetone (first 22 paintings consisted of methylcholanthrene in acetone in olive oil, 5% and 1%). At this stage B and E nipples show definite enlargement. Administration of concentrated diffusate or paintings or of both were discontinued, and nipples subsequently studied for irreversible effects (Fig. 3).

FIG. 3. Rabbit 27-07. About 3 mo after cessation of injections of concentrated diffusate of exudate in B area, enlargement of nipple has not regressed, but appears somewhat more prominent than at termination of injections (Fig. 2). Note therefore irreversible effects induced by repeated injections of proliferative factor of exudates. A similar irreversibility of effect seems to have occurred in E nipple in which repeated concentrated diffusate injections and paintings had been administered prior to cessation 3 mo before.

‡ Studies on growth factor of exudates kindly conducted by Dr. Jay Roth of Hahnemann College of Medicine are interesting. He found in 2 determinations by Orcinol reaction, an average equivalent of 0.23 mg/ml of ribose nucleotide and average of 0.27 mg/ml of nitrogen. Since about 12% of nitrogen is utilized in formation of RNA or 0.03 mg nitrogen, there is still 0.27 mg minus 0.03 nitrogen or 0.24 mg nitrogen/ml that can be used in formation of a peptide. It is doubtful that this excess nitrogen is referable to protein since the growth-promoting factor is obtained as diffusible material, and is generally Biuret negative.

the diffusate, the nipple had increased further in size. This significant observation indicates that after passage of a threshold in period and number of injections, a state of irreversibility in growth has been established. This has been observed in several rabbits and will again be described in connection with injections of a further purified fraction obtained from concentrated alkaline diffusate material by adjusting it to pH 4.3-4.6 with N acetic acid. Microscopically, months after cessation of injections, hyperplastic effects in cutaneous epithelium has also failed to regress appreciably. Furthermore, breast tissue of injected nipples, several months after cessation of injections, manifests considerable interductal fibrosis and cystic dilatation of the ducts. This is not seen in either untreated nipple or in nipple repeatedly injected with physiological saline.

B. *Thermostability of growth factor.* The factor appears heat stable. Several non-pregnant rabbits repeatedly injected in their nipples with concentrated diffusate of exudate, and also with the same material, but first brought to a boil and cooled, display no significant difference in end results. The factor therefore appears to be relatively heat stable. This is not wholly surprising since both nucleotides and polypeptides are known to be heat stable.

C. *Biological effects of active fraction recovered as growth-promoting factor of exudates.* The growth-promoting factor of exudates can be recovered as a precipitate at about pH 4.5 by adjusting the concentrated diffusate with N acetic acid. This precipitated material at times brought back to pH 7.0 with diluted NaOH, when injected into nipples of rabbits, induces marked proliferative effects and enlargement of nipples (Fig. 4, nipples C and D) (following 34 administrations for 3 months). The photograph was taken 2 weeks after ceasing administration of active growth material. Three and a half months after termination of injections the nipples not only had not regressed, but had enlarged further (Fig. 5). The growth process had evidently reached irreversibility. In nipple E (Fig. 5), 13 injections had been performed each with 0.5 ml of phosphate buffer at pH 6.2 as a further control. Repeated injections of the buffer at acid pH had no appreciable growth effect on the nipple (Fig. 5). Similar observations were made on 8 non-pregnant rabbits repeatedly injected into breast tissue with this active growth-promoting fraction of exudates.

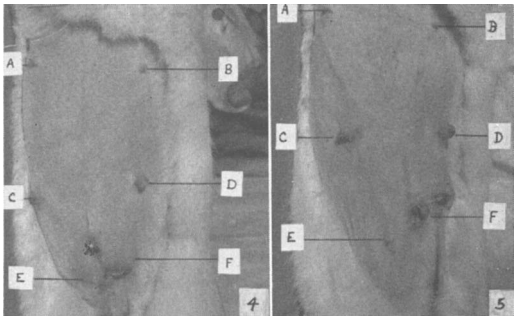

FIG. 4. Rabbit 19. Effect of repeated injections of purified fraction of growth factor of exudates on breast tissue of non-pregnant rabbit. Nipples A and B untreated. Nipples C and D inj. 34 times each with purified fraction of growth-promoting factor for 3 mo. Photograph taken 2 wk after cessation of injections. Note that nipples (particularly D) are enlarged. Nipple F inj. 21 times with crude concentrated diffusate of exudate for 2 mo. In this nipple there developed a superimposed suppurative inflammation. Nipple E inj. 13 times with phosphate buffer at pH 6.2 for one mo induced no appreciable effect.

FIG. 5. Rabbit 19. Effect of repeated injections of purified growth-promoting factor of exudates on breast tissue of non-pregnant rabbit. Nipples A and B untreated. Nipples C and D inj. 34 times each with purified growth factor for 3 mo. Three and a half mo following cessation of injections, enlarged nipples C and D failed to regress, but have enlarged further (*cf.* Fig. 4). Nipple F inj. 21 times with crude concentrated diffusate material from exudates for 2 mo. There developed also a superimposed inflammatory reaction with suppuration. In nipple E, 13 injections of phosphate buffer at pH 6.2 for one mo induced no appreciable effect.

Microscopic examination of breast tissue of a nipple repeatedly injected with this further purified fraction of growth factor of exudates reveals marked proliferative activity of the lining epithelium in the ducts (Fig. 6) (following 22 injections of the active fraction for 7 weeks). Note pseudostratification and squamous metaplasia as epithelial cells protrude into lumen of the duct. There is some, but no appreciable infiltration of chronic inflammatory cells at periphery of the duct (Fig. 6). Appearance of duct resembles chronic cystic

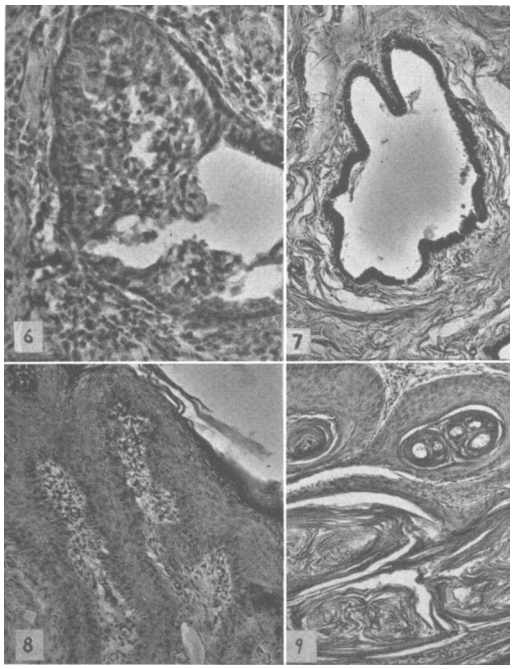

FIG. 6. Rabbit 38-91D. Appearance of a duct in breast tissue of nipple inj. 22 times with purified growth-promoting fraction of exudates over 7 wk. Note marked proliferative effect of epithelium as it protrudes into lumen. This recalls that encountered in chronic cystic mastitis of human beings. × 104.

FIG. 7. Rabbit 38-91A. Appearance of ducts in untreated breast tissue taken from same animal as in Fig. 6. Epithelial lining of ducts is normal in contrast to Fig. 6. × 75.

FIG. 8. Rabbit 38-91. Appearance of cutaneous epithelium adjacent to nipple inj. 22 times within 7 wk with purified fraction of growth-promoting factor of exudates. Note marked hyperplasia with dipping down of "tongues" of epithelial tissue containing also mitotic figures. Specificity of effect of growth factor of exudates is indicated by its absence in concentrated blood serum diffusate when latter is extracted by same procedure. Repeated injections of physiological saline also fail to induce similar proliferative effects.

FIG. 9. Rabbit 38-91. Appearance of hair follicles adjacent to nipple inj. 22 times in 7 wk with purified fraction of growth-promoting factor of exudates. Note marked hyperplasia and considerable keratinization. × 75.

mastitis of humans. This is in marked contrast to appearance of untreated breast in the same animal (Fig. 7). The ducts fail to reveal evidence of hyperplasia in the lining epithelium. Cutaneous epithelium in the vicinity of repeatedly injected nipple displays also marked epithelial hyperplasia with "tongues" of this tissue projecting deep into the corium (Fig. 8). In this actively proliferating epithelium, mitotic figures are found in moderate numbers. Adjacent hair follicles likewise reveal considerable hyperplastic effects. Keratinization is found in abundance in numerous hair follicles (Fig. 9).

Discussion. Enzymatic observations reported here indicate that growth-promoting factor of inflammatory exudates contains peptide and nucleotide structures concerned in its activity. Diffusibility, thermostability, ultra-violet absorption, and chromatographic studies of this factor are consistent with this view. Hydrolysis with trypsin inactivates biological activity of growth factor of exudates. Hydrolysis with ribonuclease likewise appreciably inactivates it. Growth factor of exudates in its further purified state displays striking proliferative effects both on breast tissue and on adjacent cutaneous epithelial structures.

The presence of this factor in exudates offers a reasonable explanation for the mechanism of repair following inflammatory reaction. It is conceivable that the factor in its present state or in a more purified form may have practical application in wound healing. This aspect is being studied. Continuous chemical studies to determine the precise nature of growth-promoting factor of exudates are desirable. The possibility of its being a nucleopeptide is merely suggestive, and is being studied. Growth factor of exudates is specific in causing proliferative responses, for when blood serum is substituted in the extracting procedure the final fraction displays no proliferative effectiveness(1,2,9). As shown earlier, repeated utilization of an acute inflammatory irritant such as croton oil in blood serum fails to induce proliferative effects(9). Repeated saline injections likewise yield no proliferation(2). When, however, a protracted chronic inflammation leads to a proliferative state, it is conceivable that growth-promoting factor is liberated by injured cells at the site of inflammation.

Isolation of a growth factor from inflammatory exudates may also aid in further elucidation of the role of inflammation in carcinogenesis. The evidence indicates that repeated administration of the factor produces not only

progressive growth of breast tissue in non-pregnant rabbits, but also, after 30 to 35 injections irreversibility occurs whereby the enlarged nipple not only fails to regress in size, but increases, though administration of growth factor has been discontinued (*cf.* Fig. 3, 4, 5, 6). It is unlikely, however, that growth factor *per se* can induce a cancer. It is more likely, as pointed out elsewhere, that it acts as a cocarcinogen in accelerating the effect of a carcinogen, whether of exogenous or of endogenous origin(4). The latter may in turn be liberated as a result of somatic mutation or genetic susceptibility. This may be the real significance of the growth factor of exudates in evaluating the precise role of a long standing inflammation in carcinogenesis.

This growth factor may help to clarify how a long standing inflammatory response sometimes gradually develops into a neoplasm (1,2). It is possible that the diverse ways in which neoplasms can be induced may be in part explained in terms of a final common pathway, and this pathway may be through liberation of a growth-promoting substance by mildly injured cells(1,2,3,4,9). This type of injury may be the result of chronic irritation, virus infection, or hormonal imbalance(9). However, there is little doubt that the growth factor released by injured cells is in itself insufficient to induce development of a neoplasm. Initiation of the process by some unknown genetic mechanism seems of primary importance. Liberation by a variety of means of the endogenous growth factor from injured cells seems only to act as a further promoter or cocarcinogen to a more fundamental process.

Summary. There is present in inflammatory exudates a diffusible growth-promoting factor which can be purified further from concentrated diffusate by precipitation at pH 4.5 with N acetic acid. Repeated injections of the fraction in its present state of purification induce in non-pregnant rabbits conspicuous hyperplasia of epithelial structures as well as a condition in breast tissue simulating that seen in chronic cystic mastitis. A state of irreversibility is established upon termination of a prolonged course of injections of growth-promoting factor of exudates. The enlarged nipples fail to regress in size, though administration of the active material is discontinued. By enzymatic inactivation, evidence is advanced that both nucleotide and peptide structures appear to play a role in biological activity of the growth-promoting factor of exudates.

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