IN VITRO CONTROL OF THE DEVELOPMENT OF MACROPHAGE AND GRANULOCYTE COLONIES

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In order to analyze the control mechanism that regulates the growth and differentiation of normal cells from hemopoietic organs, it would be desirable to be able to study the entire process in clones in tissue culture. It was shown in our previous studies on the development of such an *in vitro* analysis[1, 2] that two types of colonies can be obtained in culture by seeding spleen cells from normal adult mice in a soft agar medium in the presence of a feeder layer of unirradiated or X-irradiated normal mouse embryo cells seeded underneath the agar. One type of colony contained cells provisionally classified on the basis of their morphology in agar as "mast" cells, and the other type contained neutrophile granulocytes. It was also shown that the feeder cells release into the medium a nondialyzable substance with a high degree of thermostability that can induce, in the absence of the feeder layer, the multiplication of colony-forming cells and the formation of "mast" cell clones from single cells.[2] The present experiments are concerned both with the induction and the inhibition of these two types of colonies.

Materials and Methods.—Cell cloning: The cells used for cloning were from the spleens of normal 60–80-day-old males from the inbred mouse strain SWR, or from embryo livers taken at about the 17th–19th day of gestation from an inbred strain of Swiss mice. The adult spleens were washed in phosphate-buffered-saline (PBS), teased with forceps to make a cell suspension, washed with PBS, and then diluted in PBS to an appropriate cell number for cloning. The embryo livers were made into a cell suspension with scissors and light pipetting in serum-free Eagle's medium with a four-fold concentration of amino acids and vitamins (EM). This suspension was then diluted in EM after 5 min standing to allow aggregates to sediment, and a sample of the cells used for cloning was counted in eosin solution at a final eosin dilution of 1:2000. Cells were cloned in 50-mm plastic Petri dishes (Falcon plastics) in soft agar on a harder agar base as described previously.[1, 2] EM supplemented with 20% inactivated (56°C for 30 min) horse serum was used as the medium for both agar layers. Liver cells were normally seeded for cloning at 3×10^4 cells per plate and spleen cells at 1×10^6 cells per plate. Colonies were counted microscopically after the seeding of embryo liver cells, and on a bacterial colony counter with a ×2 magnifying glass after seeding of adult spleen cells. In the microscopic scoring, more than 50 cells were counted as a colony. Each point in an experiment was based on the results from 2–4 plates. The feeder cells, when present, were underneath the lower agar layer. Conditioned medium was added to the lower agar layer, and dilutions of conditioned medium were made so that there was always the same percentage of new EM plus 20% horse serum.

Cells used as feeder layers and for the production of conditioned medium: Cultures of cells used as feeder layers or for the production of conditioned medium were grown in EM supplemented with 10% inactivated horse serum. Unless otherwise stated, the normal cells were obtained from SWR or Swiss mice; secondary embryo cultures were seeded at 2×10^5 cells per 50-mm plate; kidneys from 6–10-day-old mice at one kidney per plate and from 30–40-day-old mice at 2.5×10^5 cells per plate; adult spleen at 5×10^7 cells; and peritoneal cells at $1–2 \times 10^6$ cells per plate. Peritoneal macrophages were obtained from 2–3-month-old SWR males by intraperitoneal inoculation of 5 ml PBS at 4 days after intraperitoneal inoculation of 3 ml thioglycolate medium.[3] The peritoneal cells were washed twice in PBS before seeding, and the macrophages used as feeder layers after 1–2 days in culture. A mouse cell line established *in vitro* from Swiss embryo cells, which will be referred to as E1, was also tested for inductive capacity. Unless otherwise stated, conditioned medium was used after centrifugation at 650 *g* for 10–15 min, and was stored before use at 4°C

or −20°C. Cells used as feeder layers or for conditioned medium were usually from the same inbred mouse strain as the cells used for cloning.

Cell staining and number of cells per colony: For cell staining, colonies were removed from the agar with a thin spatula and smeared on glass slides. After drying, the slides were stained with May-Grünwald-Giemsa. To determine the number of cells per colony, 30 or more pooled colonies were suspended in 1 ml PBS and counted in a hemocytometer.

Results.—The development of granulocyte and macrophage colonies: Of the two types of colonies previously observed after seeding adult spleen cells,[1, 2] one type contained cells ranging from myeloblasts to neutrophil granulocytes and these will be referred to as granulocyte (G) colonies. The other type contained cells with many large metachromatic granules provisionally referred to on the basis of their morphology in agar as "mast" cells.[1, 2] In order to determine whether the metachromatic granules in these cells were induced by their growth in agar,[4] spleen cells were seeded with 25 per cent conditioned medium from secondary embryo cell cultures in 7 ml of 1.3 per cent methyl cellulose[5] (Methocell 4000 cps, Dow Chemical Co.), and in 7 per cent hydrolyzed starch (Connaught Medical Research Labs.) on a 10 per cent starch base layer. Colonies were obtained, but the cells morphologically like "mast" cells in agar did not contain the many large metachromatic granules in these substrates (Fig. 1). These cells are thus more probably macrophages, and this type of colony will be referred to as macrophage (M) colonies.

The seeding of embryo liver cells with secondary embryo feeder layers gave a higher number of colonies than the seeding of adult spleen. A microscopic analysis of the types of colonies obtained at various times after seeding embryo liver cells showed that there were colonies of M, G (Fig. 1), mixed M and G, and blast (B) cells. The G colonies contained cells in various stages of differentiation, from myeloblasts to neutrophile granulocytes, whereas the B colonies mainly contained cells classified as myeloblasts. The number of B, G, and mixed M and G colonies decreased from 5 to 15 days after seeding, whereas the number of M colonies increased during the same time (Fig. 2). Colonies of degenerate cells were observed from the 7th day. The B colonies presumably degenerated early without further differentiation or differentiated to G colonies. The average cell number of the G colonies at 5, 7, 19, and 15 days was 430, 3,560, 7,250, and 19,890 (average of three colonies), respectively. Blast cells were always found in the larger G colonies observed at about 10–15 days after seeding, but not in the G colonies that degenerated earlier. This indicates that the early-degenerating G colonies were probably derived from myelocytes or metamyelocytes, whereas the larger G colonies were probably derived from myeloblasts that continued to divide before differentiating to granulocytes. In the mixed M and G colonies, the M cells were on the outside. The M colonies contained the largest cells among the three types of colonies, and they were usually not as compact as the G and B colonies.

It has been shown that M colonies can be derived from single cells.[2] The seeding of different numbers of liver cells on feeder layers of E1 cells (see below) showed a linear relationship between number of cells seeded and number of G colonies, indicating that a G colony can be initiated by a single colony-forming unit. Attempts to re-clone G colonies have so far not been successful. It can reasonably be assumed that the mixed M and G colonies arose from admixture of cells of the two types of colonies, although it is still to be determined whether M and G colonies can originate from the same cell.

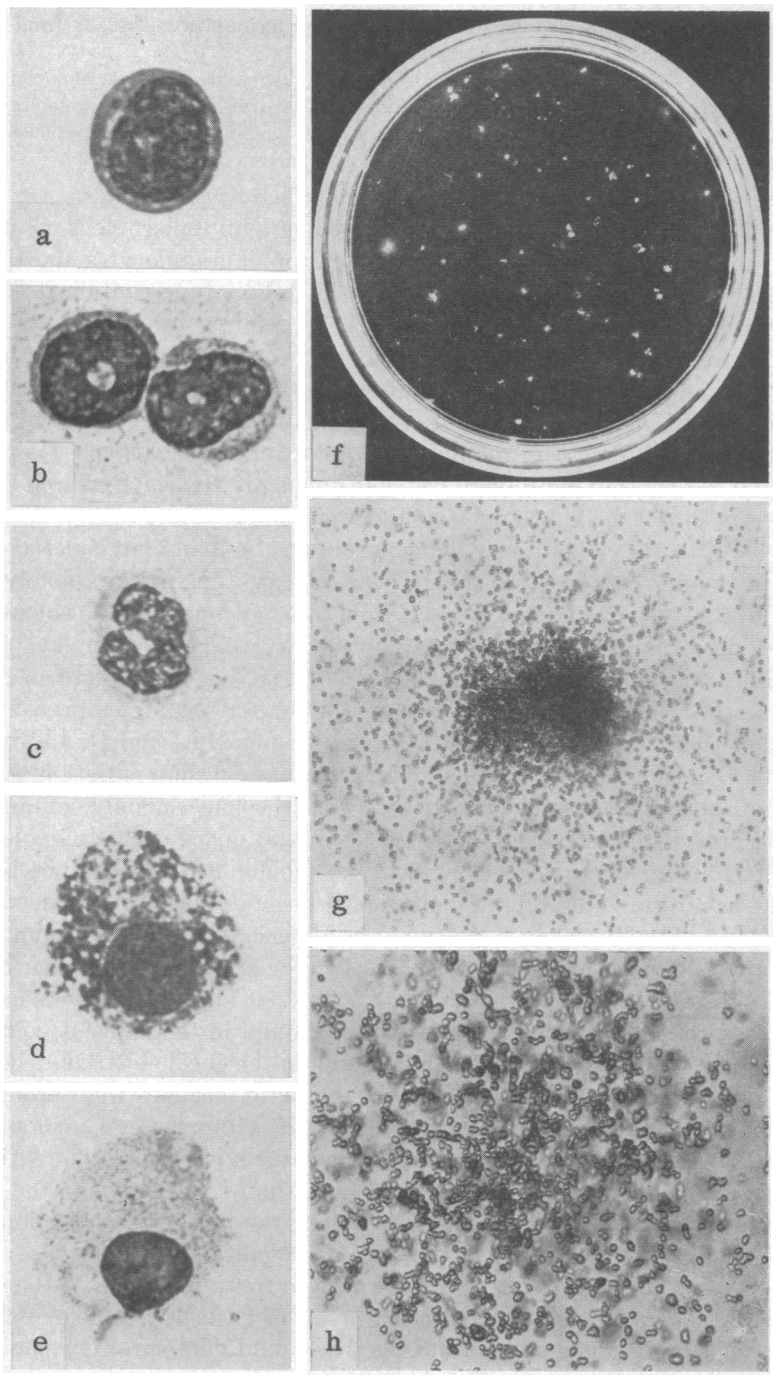

FIG. 1.—(*a–c*) Some stages in the differentiation of granulocytes in a granulocyte colony in agar. (*d*) Macrophage with many large granules, in agar. (*e*) Macrophage without the many large granules, in starch. (*a–e*) Eleven days after seeding adult spleen cells, (*a–c*) with embryo cell feeder layer, and (*d*) and (*e*) with 25% embryo cell conditioned medium. The cells were stained with May-Grünwald-Giemsa. All ×1400. (*f*) Colonies in agar from adult spleen cells on embryo cell feeder layer. ×1. (*g*) Granulocyte colony, and (*h*) macrophage colony, from embryo liver cells in agar on embryo cell feeder layer. Both ×60; (*f*), (*g*), and (*h*), unstained.

Induction by feeder layers and conditioned medium from embryo, kidney, and the cell line E1: In experiments with embryo liver cells on the inductive capacity of different feeder layers, one batch of kidney feeder layers from 10-day-old mice used at 3 or 5 days after cell seeding gave a majority (72 and 86%) of M colonies at 7 days; whereas another batch of kidney feeder layers from 6-day-old mice used at 3 days after cell seeding gave a majority (95%) of G and B colonies. Evidence supporting the assumption that this may reflect a difference in inductive capacity for M and G colonies by different cell types was obtained in that feeder layers of the line E1 gave almost only G colonies (Table 1).

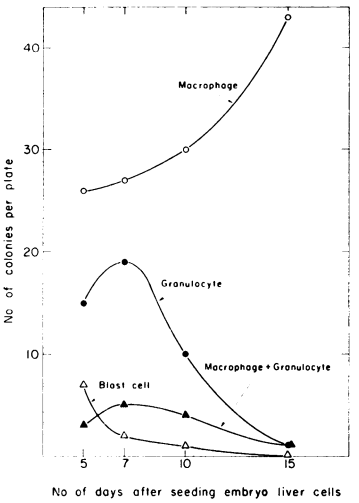

FIG. 2.—Number of macrophage, granulocyte, mixed macrophage and granulocyte, and blast cell colonies, at different times after seeding 3×10^4 embryo liver cells with secondary embryo cell feeder layers.

That inducing substance can be produced by cells from adults was shown by a comparison of a 50 per cent concentration of 7 days' cumulative conditioned medium from cultures of secondary embryo cells, kidneys from 7–10-day-old mice, and kidneys from 30–40-day-old mice. The cloning efficiency was one colony per 550, 510, and 380 cells seeded, respectively, and the colonies counted at 10 days after seeding were mainly M colonies.

Although a 50 per cent concentration of conditioned medium gave mainly M colonies, there was an increase in the per cent of G colonies with an increase in dilution with 7 days' cumulative conditioned medium from embryo cells, and 3 or 4 days' cumulative medium from kidneys from young animals. The results from two experiments with kidney cells are shown in Figure 3. The data indicate that at the higher dilutions of the conditioned medium from embryo and kidney cells, there was a diluting-out of an inhibitor for G colonies. In contrast to the finding of almost only G colonies when cells of the line E1 were used as feeder layers (Table 1), 7 days' cumulative conditioned medium from E1 gave both M and G colonies, with a majority of M (Table 2). With E1 cells, the use of conditioned medium presumably either diluted out or inactivated an inhibitor for M colonies. This medium showed no increase in the percentage of G colonies with an increase in dilution.

It should be mentioned that inducing substance for M colonies was found in

TABLE 1

INDUCING ACTIVITY OF FEEDER LAYERS OF SECONDARY EMBRYO CELLS AND THE CELL LINE E1

Feeder layers		No. of Days after Seeding Liver Cells 7	10	15
Cell line E1	Total colonies per plate	41	23.0	2.3
	G colonies per plate	38	22.8	2.0
		(93%)	(99%)	(87%)
Secondary embryo cells	Total colonies per plate	52	44	45
	G colonies per plate	19	10	1
		(37%)	(23%)	(2%)

Embryo liver cells were seeded at 3×10^4 cells per plate. Feeder cells were seeded at 2×10^5 cells per plate and used 2 days later. The cell line E1 was used at the 14th passage.

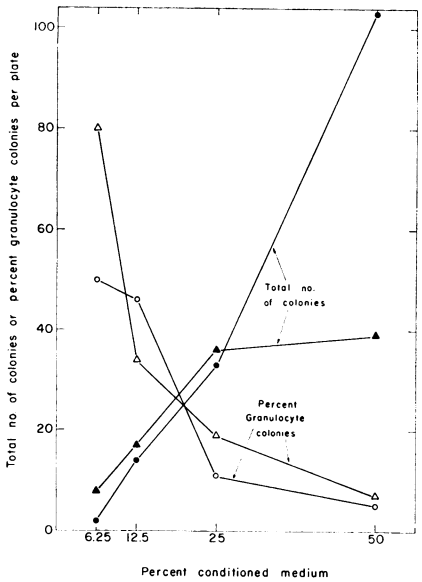

FIG. 3.—Total number of colonies and % granulocyte colonies obtained with different conc. 3 or 4 days' cumulative conditioned medium from baby kidney cultures. Results from two expts.; 3×10^4 embryo liver cells seeded per plate, and colonies counted at 7 days.

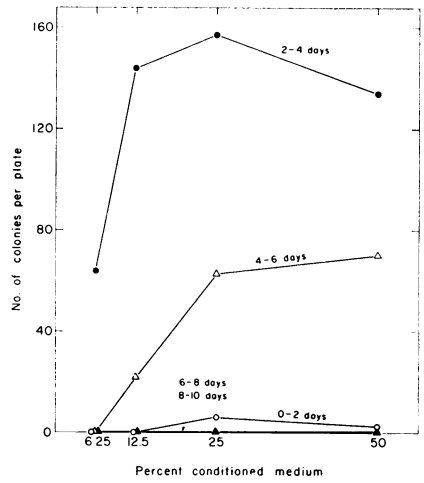

FIG. 4.—Inducing activity of conditioned medium taken at different times from mass cultures of cells from adult spleen. Mass cultures were seeded at 5×10^7 cells per plate, medium was changed every 2 days, and inducing activity tested by seeding 3×10^4 embryo liver cells per plate. Colonies counted at 10 days.

conditioned medium from the cells of three transplanted mouse tumors tested with adult spleen, and counted at 13 days after seeding. This conditioned medium was tested at a concentration of 50 per cent or less, and for two of the tumors (one induced by polyoma virus and the other by methylcholanthrene in C57B1/6 mice), it showed the same inducing capacity as conditioned medium from C57B1/6 or SWR secondary embryo cells, whereas for the third tumor (induced by polyoma virus in a SWR mouse), it showed a lower inducing activity.

Induction by conditioned medium from spleen cells: The inducing capacity of conditioned medium from large numbers of cells from adult spleen was tested by seeding 5×10^7 cells per plate as mass cultures. The medium was changed every 2 days, and tested for its inductive capacity with embryo liver cells. The results (Fig. 4) show that there was inducing activity in samples taken up to 6 days, with a peak of activity in the 2–4-day sample, but no induction with the 6–8- and 8–10-day medium. At all dilutions of the conditioned medium tested, the colonies were 99–100 per cent M colonies at the tenth day. These results indicate that some cells in the adult spleen can produce the substance that induces M colonies. There was a change in the cell population of the mass cultures at various times after seeding. Many lymphoid cells were present until 4 days. For the 6–8- and 8–10-day medium, the cell population consisted predominantly of macrophages, thus indicating that the macrophages present at these times did not produce the inducing substance. It was also found that conditioned medium and feeder layers of peritoneal macrophages, and feeder layers from adult spleens consisting of macrophages, did not induce the formation of M or G colonies.

TABLE 2

COMPARISON OF INDUCING ACTIVITY OF FEEDER LAYERS AND CONDITIONED MEDIUM FROM SECONDARY EMBRYO CELLS AND THE CELL LINE E1

Type of colonies	Feeder Layers: Cell line E1	Feeder Layers: Secondary embryo cells	Concentration of Conditioned Medium: Cell Line E1 50%	Cell Line E1 25%	Cell Line E1 12.5%	Cell Line E1 6.25%	Secondary Embryo Cells 50%	Secondary Embryo Cells 25%	Secondary Embryo Cells 12.5%	Secondary Embryo Cells 6.25%
M	0	44	18	23	22	7	35	24	7	1
G	28	18	10	8	9	3	3	5	4	1
B	3	2	1	2	1	1	2	3	3	0
Mixed M and G	0	6	6	6	3	1	2	3	1	0

Embryo liver cells were seeded at 3×10^4 cells per plate and the colonies counted at 7 days. Feeder cells were seeded at 2×10^6 cells per plate and used 2 days later. The results with feeder layers are the averages of three experiments with the 15th, 18th, and 19th passages of E1 cells, and the averages of two experiments with secondary embryo cells. Conditioned medium was 7 days' cumulative medium obtained after seeding 1×10^6 cells per 100-mm plate, and the results are averages of two experiments (17th and 21st passage) with E1, and of three experiments with secondary embryo cells.

Inhibition by macrophages: As no colony induction was observed with feeder layers or conditioned medium from these spleen or peritoneal macrophages, the question arose whether these cells could inhibit the action of inducing conditioned medium produced by other cells. The results of experiments to test this showed that feeder layers of spleen cells that consisted predominantly of macrophages at the end of the experiment, and feeder layers of peritoneal macrophages, completely inhibited colony formation by 6–50 per cent conditioned medium from secondary embryo cells. As a check on the specificity of this inhibition, cells from two polyoma and one benzo(a)pyrene-induced mouse tumor were cloned in agar with a feeder layer of peritoneal macrophages. In all cases the number of colonies was the same as in the controls, but there was some reduction in colony size.

Some properties of the inducing substance for macrophage colonies in conditioned medium: Some further properties of the inducer for M colonies in conditioned medium from embryo cells[2] were tested with adult spleen cells. A high inducing activity was found in 1- or 2-day samples taken between 4–8 days after seeding secondary embryo cells. When the medium was changed to serum-free EM after 3 days in EM plus serum, the inducing activity of 4- or 5-day serum-free medium was as high as that of 2-day medium with serum (Table 3). Centrifugation of conditioned medium with serum from secondary embryo cells showed that there

TABLE 3

INDUCING ACTIVITY FOR MACROPHAGE COLONIES BY CONDITIONED MEDIUM FROM SECONDARY EMBRYO CELLS AFTER CENTRIFUGATION AT 50,000 *g*, AND WITHOUT SERUM

Conditioned medium	Part of centrifuge tube	Time of centrifugation (hr)	Concentration of Conditioned Medium 50%	25%	12.5%	6.25%
			Colonies per Plate			
After centrifugation at 50,000 *g*	Top half	2	121	94	85	18
	Bottom half	2	124	148	97	27
	Top half	17	78	74	35	2
	Bottom half	17	108	103	96	52
	Pellet	2 or 17	0	0	0	0
Control	—	0	116	82	56	42
Without serum	4 days' cumulative		45	21	0	0
	5 days' cumulative		51	33	0	0
Control with serum	2 days' cumulative		57	31	6	0

Adult spleen cells were seeded at 1×10^6 cells per plate and colonies counted macroscopically at 11–13 days; 11 ml of 7 days' cumulative medium from secondary embryo cultures was centrifuged at 50,000 *g* in a Spinco type 40 rotor. The pellet from 11 ml conditioned medium was resuspended in 10 ml EM. For the experiment with serum-free medium, secondary embryo cultures were grown in EM plus 10% horse serum for 3 days, and the medium was then changed to EM without serum.

was still a high inducing activity in the supernatant after 17 hr centrifugation at 50,000 *g* (Table 3). For further experiments with the serum-free medium, conditioned medium was centrifuged at 50,000 *g* for 3 hr before use. The inducing activity of this medium for M colonies was not destroyed after incubation for 3 hr at 37°C with 200 γ/ml of crystallized trypsin or chymotrypsin (Worthington Biochemical Corp.), and pronase (Calbiochem.), and incubation for 18 hr at 37°C with 200 γ/ml collagenase (Worthington). Incubation for 3 hr with 2 mg/ml trypsin followed by ½ hr incubation with 6 mg/ml soybean trypsin inhibitor (Worthington) did not make the inducing substance dialyzable. The results of ammonium sulfate fractionation have shown that the inducing activity was completely precipitated in 33–50 per cent ammonium sulfate, and that there was no inducing activity in the 0–33, 50–65, 65–100 per cent, or supernatant fractions.

Discussion.—The present results indicate that conditioned medium contains inducer and inhibitor for M and G colonies, and that different types of feeder cells produced different amounts of these substances. It can be postulated from the results with feeder layers of macrophages that inhibition of the activity of the inducer by macrophages can serve as a control mechanism for the production of M and G cells. It will be of interest in further studies to determine whether the same inducer can act on both types of colonies, whether the two types of colonies can be produced from the same cell by two different inducers, and if inducer penetrates the target cell or acts at the cell surface. The high degree of thermostability of the nondialyzable inducer for M colonies[2] and the activity of the supernatant after centrifugation at 50,000 *g* for 17 hr differentiate this inducer from the substance in conditioned medium[6] that can produce the growth-enhancing effect of feeder layers for different types of cells.[7] In addition to the difference in the nature of the target cells, the results showing inducing activity after incubation with trypsin, chymotrypsin, pronase, and collagenase also suggest that the M colony inducer differs from the nerve[8] and epithelial[9] growth substance, and from the substance that supports the development of clones of muscle cells.[10] The inducer for M colonies was also not inactivated by ribonuclease.[2] The number of different inhibitors involved in the present system, and their relationship to the substance that inhibits the growth of small numbers of embryo cells,[6] remains to be elucidated.

Summary.—Feeder layers and conditioned medium from certain types of mouse cells can induce *in vitro* the development of macrophage (M) and granulocyte (G) colonies from cells of adult mouse spleen or embryo liver. G colonies contain cells in various stages of differentiation, from myeloblasts to neutrophil granulocytes. When tested with embryo liver, feeder layers and conditioned medium from embryo and kidney cells can induce M and G colonies, and an increase in dilution of the conditioned medium resulted in an increase in the percentage of G colonies. Feeder layers of a mouse cell line established *in vitro* induced almost only G colonies, whereas conditioned medium from cells of this line induced a majority of M colonies. Spleen and peritoneal macrophages inhibited the inducing effect of conditioned medium from embryo cells. The results indicate that inducer and inhibitor for the development of M and G colonies can be produced by feeder cells *in vitro*, and that different feeder cells produced different amounts of these substances. It is suggested that inhibition of the activity of inducer by macrophages can serve as a control mechanism for the production of M and G cells. The inducer tested with

M colonies, which is nondialyzable and has a high degree of thermostability, was not destroyed by incubation with trypsin, chymotrypsin, pronase, or collagenase, and was precipitated with 33–50 per cent ammonium sulfate.

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