QUANTITATIVE METHOD FOR MEASURING ADJUVANT-INDUCED GRANULOMA ANGIOGENESIS IN INSULIN-TREATED DIABETIC MICE

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The angiogenesis of adjuvant-induced pouch granuloma was studied in insulin-treated diabetic mice by a newly established method using carmine dye. The 10% carmine suspension in 5% gelatin solution was infused through the tail vein of mice to be distributed to the end of the capillaries in the granulation tissue without leakage. The carmine dye was extracted from the tissue with 3 N NaOH solution and then measured by spectrophotometry. The content of carmine dye in the granuloma tissue in alloxan diabetic mice was observed to be significantly low during the first week after adjuvant injection when compared with normal mice, indicating poor development of blood vessels in the diabetic state. Diabetes-induced inhibition of the angiogenesis was completely restored by the treatment with insulin in a dose producing no hypoglycemic effect. These results were directly reflected by the formation of granuloma tissue. This method was established to be explicitly useful for measuring the angiogenesis, especially in mouse granuloma tissue.

Keywords — mouse pouch granuloma; angiogenesis; carmine dye method; alloxan diabetes; insulin treatment

INTRODUCTION

The adjuvant-induced pouch granuloma in mice has been suggested to be a beneficial experimental model to measure the drug effects on angiogenesis, because the blood vessel organization in this granuloma is not as complicated as that of the rat.¹⁾ In the mouse granuloma tissue, many vessels were observed to be formed by Freund's complete adjuvant with 0.1% croton oil. Thus, an aim of this study was to establish a quantitative method for adjuvant-induced granuloma angiogenesis. The developing vessels were measured by the increasing amount of vital dyes. As for vital dyes used, carmine was found to be the most suitable, compared with other dyes including Evans blue dye.

This newly established method was applied to investigate granulomatous tissue in the diabetic state. In experimental diabetes the reduction of granuloma formation is globally reported.^{2,3)} In genetically diabetic KK-CA^y mice and alloxan diabetic mice, a similar reduction was also reported, which was found to be reversed by insulin treatment.^{4,5)} However, more detailed findings on angiogenesis have not been obtained as yet, despite this important factor in granuloma formation.

In the present paper, we describe a quantitative method to measure angiogenesis in granuloma produced in insulin-treated diabetic mice.

MATERIALS AND METHODS

Animals and Materials — Male ICR mice, each weighing 25–30 g, were purchased from Japan CLEA (Tokyo) and kept in a room maintained at a constant temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$), and illuminated from 7 am to 7 pm. Freund's complete adjuvant kit, crystalline insulin and alloxan monohydrate were obtained from Sigma, Evans blue dye and carmine dye from Merck, and croton oil, gelatin, methylsalicylate and other chemical reagents for dye extraction and preparing transparent specimens from Junsei Pure Chem. (Tokyo).

Adjuvant Pouch Method and Drug Application — The adjuvant pouch was prepared as described previously.¹⁾ Air (3 ml) was subcutaneously injected into the loose connective tissue on the back of the mice. Soon after the air-pouch was made, alloxan monohydrate was injected into the tail vein at a single dose of 60 mg/kg of body weight and Freund's complete adjuvant with 0.1% croton oil was injected into the pouch 24 h later.

Insulin (20 U/kg) or normal saline was administered daily into the adjuvant pouch in normal and alloxanized mice from the day of the adjuvant injection and 10 mice in each group were sacrificed on day 3, 5, 7 and 14.

Quantitative Measurement of Newly Formed Blood Vessels (Carmines Dye Method) — Using the carmine dye infusion method of Okuhira *et al.*,⁶⁾ which has been used for angiocardiology, the amount of carmine dye circulating in newly formed vessels in the granuloma tissue was measured. One ml of a carmine suspension (10% w/v) in 5% gelatin solution warmed to 40 °C was injected into the tail vein of each mouse. After the gelatin was solidified by cooling the cadaver below 4 °C for a few hours, the granuloma tissue was removed. The tissue for each mouse was dissolved in 5 ml of 3 N NaOH solution for 30 min at room temperature and then completely digested in hot water (50 °C) for 10 min. The digested samples were centrifuged at 2 500 rpm for 15 min and filtered through 0.45 μ m of millipore-filter. The filtrates were measured spectrophotometrically at 530 nm against a blank tissue specimen obtained without infused carmine.

Preparation of Transparent Specimens — Half-cut pouch walls were fixed with 100% ethanol for more than 5 d at below 4 °C. After complete dehydration, the tissue specimens were soaked with methylsalicylate for more than 2 d. The established transparent specimens were observed under a Nikon Multiphoto-109 type microscope.

Statistical Analysis — The data were analyzed statistically by the *t*-test, and the criterion for statistical significance was the 0.05 level.

RESULTS

Methods of Infusing Evans Blue Dye and Carmine Dye

We first examined the method of infusing Evans blue dye. After infusion of one ml of Evans blue dye (1% w/v), the dye quickly leaked from the capillaries into the extracellular space of the granuloma tissue (Fig. 1A). This did not support the postulation⁷⁾ that the amount of dye in the granuloma tissue depends on the volume of blood vessels in it, but it mainly depends on the duration of infusion. Namely, the granuloma tissue was dyed blue during only 2 min.

In contrast, the intravenously injected car-

FIG. 1. *Newly Formed Blood Vessels in the Adjuvant-Induced Granuloma Pouch in Mice Sacrificed after Dyes Infusion*

A. Infusion of Evans blue dye for 2 min. Note that the pouch wall has been dyed blue by leakage. B. Infusion of carmine dye suspended in gelatin solution. Granuloma tissue has been isolated and its transparent specimen was observed (magnifying $\times 3$).

mine dye did not leak and all of it remained in the vessels from their larger parts to the end of the capillaries (Fig. 1B). The time course experiment demonstrated that the vessels were gradually developed from day 3 to 7 as shown in Fig. 2. All these nascent vessels in granuloma tissue sprouted from the vessels existing in skin and dorsal muscles and developed with the granuloma formation.

Quantitative Measurement of Carmine Content in Granuloma Tissue

The calibration curve and recovery of the carmine dye were investigated. Figure 3 shows the standard curve of carmine dye for concentrations of 0.03 to 1.0 mg/5 ml of 3 N NaOH solution per tube containing a granuloma pouch. The turbidity in the sample solution was removed by filtration with a millipore-filter and color contamination was negated by using the granuloma tissue without carmine as a blank. The curve was linearly related to the amount of carmine dye in the range of 0.125 to 1.0 mg/tube. In addition, the average recovery in all concentrations ranged from 95 to 103%, and the measurement error was within 5%.

Effect of Insulin on Granuloma Tissue Formation and Angiogenesis in Diabetic State

The angiogenesis in the granuloma tissue was measured by the infusion of a carmine dye. Table I shows the time course of changes in the

FIG. 2. *Transparent Specimens of Pouch Granuloma Induced by Freund's Complete Adjuvant with 0.1% Croton Oil in the Mice*

Mice were treated with carmine dye and sacrificed on day 3 to 14. Specimens were prepared as described in Materials and Methods. Scales are 5 and 2 mm in the upper and the lower photographs, respectively.

granuloma weight and the carmine content in normal and diabetic mice. Granuloma formation

and carmine content parallelly increased during the first 7 days after adjuvant injection both in

FIG. 3. *Calibration Curve between Carmine Concentration and the Degree of Absorbance (530 nm) Used in This Study*

FIG. 4. *Effect of Diabetes on Granuloma Formation and the Angiogenesis*

○, □, normal mice; ●, ▨, alloxan diabetic mice. a) $p < 0.05$, compared with normal mice.

TABLE I. *Effect of Insulin on Pouch Granuloma Weight and Carmine Dye Content in Normal and Diabetic Mice*

Mice	Days	Granuloma wet wt. (g)	Carmine content (mg)
Normal	Saline	3	0.256 ± .015 ^{a)}
		5	0.489 ± .043
		7	0.717 ± .032
		14	1.000 ± .042
	Insulin (20 U/kg)	3	0.710 ± .034
		5	0.276 ± .018
		7	0.528 ± .024
		14	0.852 ± .034 ^{b)}
Diabetic	Saline	3	0.436 ± .015
		5	1.051 ± .058
		7	0.332 ± .027
		14	0.752 ± .057
	Insulin (20 U/kg)	3	0.234 ± .014
		5	0.407 ± .041
		7	0.615 ± .020
		14	0.894 ± .026

Insulin or saline was injected daily into the pouch during the experiment periods. a) Mean ± S.E. of 10 mice. b) $p < 0.05$, c) $p < 0.01$, compared with each saline group.

normal and diabetic mice. In alloxan diabetic mice, however, both parameters were significantly smaller than those in normal mice, which indicates that they were equally influenced by the diabetic state (Fig. 4). This effect was more prominent in the severe diabetic mice (data not shown).

Insulin treatment reversed the inhibition of the formation of granuloma tissue and blood vessels (Table I). Figure 5 shows the effect of insulin (20 U/kg) injected into the pouch on the angiogenesis. In diabetic mice, insulin treatment strikingly increased angiogenesis without hypoglycemic effect, but not so remarkably in normal mice. Insulin had the greatest effect on angiogenesis on day 3 to 5, which was before it enhanced granuloma formation (day 7).

DISCUSSION

The development of blood vessels in the granuloma tissue has been measured by the contents of infused vital dyes such as Evans blue,⁷⁾ pontamine blue,⁸⁾ and pelikan ink.⁹⁾ However, the dye contents have not been precisely measured using such methods because the dyes leak out into the

perivascular spaces. After many trials searching the impermeable dye, we have established a method for measuring the development of newly formed vessels in the granuloma tissue using the carmine dye suspended in a gelatin solution. The advantages of the carmine dye method are that the dye: 1) reaches to the end of newly formed vessels, 2) does not leak out into the granulation tissue, and 3) does not change within the vessels.

Generally, the newly formed vessels are considered to be formed by sprouting, although sprouting may not be the only mode of angiogenesis.⁹⁾ The increase of vascular tissue may also be caused by the intercalation of young cells into the wall of pre-existing vessels.¹⁰⁾ In any case, carmine dye can serve as a marker of angiogenesis, which is fully supported by its distribution into all vessels in the time course experiment (Fig. 2).

In alloxan diabetic mice, on the other hand, angiogenesis was suppressed and the suppression was reversed by treatment with insulin. Since blood glucose levels did not change during this period, the insulin effect was considered not to

FIG. 5. Effect of Insulin Treatment on the Angiogenesis in Normal and Alloxan Diabetic Mice
○, saline; ●, insulin (20 U/kg) intra-pouch treatment. a) $p < 0.05$, b) $p < 0.01$, compared with saline treated mice.

be indirect, based on the lowering of blood glucose, but to be a direct factor in cell growth. The close interaction between angiogenesis and granuloma formation was also evidenced in this study because an apparent parallelism between both parameters was observed in a diabetic state with or without insulin treatment.

In conclusion, a newly established carmine dye method was demonstrated to be useful for measuring angiogenesis in granuloma tissue formation, even in the diabetic state.

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